UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

Genetic and metabolic regulation of fatty acid deposition in autochthonous bovine breeds with distinct genetic background

Ana Sofia Henriques da Costa

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"That is the essence of science: ask an impertinent question, and you are on the way to a pertinent answer."

Jacob Bronowski

À minha família

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Abstract

Genetic and metabolic regulation of fatty acid deposition in autochthonous bovine breeds with distinct genetic background

During the finishing phase, bovines deposit large amounts of subcutaneous and visceral fats resulting in production inefficiencies affecting, in particular, meat quality. Intramuscular fat composition of ruminant meats influences the quality of the final product, which explains the importance of assessing meat fatty acid profile using different breeds and feeding strategies. On the other hand, both genetic background and finishing system may alter fat deposition, indicating their influence on adipogenic and lipogenic factors. Nevertheless, the molecular mechanisms underlying fat deposition and fatty acid composition in beef cattle are not fully understood. The present study aimed to assess the effect of breed and diet on carcass composition, the cellularity of two fat depots (subcutaneous and mesenteric adipose tissues) and their implications on the fatty acid profile and the expression levels of adipogenic and lipogenic key factors and enzymes. Forty bulls from two genetically diverse Portuguese bovine breeds, Alentejana and Barrosã, were selected. The animals were fed isoenergetic and isonitrogenous diets, which were either low or high in silage percentage, until 18 months of age. Under these experimental conditions, it was shown that the genetic background is a major determinant of carcass composition and meat quality, and that the dietary differences studied had a limited effect on carcass composition. Data revealed that the subcutaneous adipose tissue has larger adipocytes than the mesenteric adipose tissue. Overall, the results showed that the fat depots' fatty acid composition is mostly dependent on the genetic background. Dietary silage level impacted on muscle lipid metabolism to a greater extent than on that of subcutaneous adipose tissue, as evaluated by levels of gene expression of adipogenic and lipogenic factors. Moreover, hepatic desaturation and elongation of polyunsaturated fatty acids was shown to be influenced by diet composition, possibly through the expression of genes encoding for enzymes associated with desaturation/elongation pathways. In sum, these findings highlight the importance of taking into account the genetic background while devising feeding strategies to manipulate the fatty acid composition of beef cattle tissues.

KeyWords: bovine; fatty acid composition; gene expression; adipogenesis; lipogenesis; fat partitioning.

Resumo

Regulação genética e metabólica da deposição de ácidos gordos em raças bovinas autóctones com fundo genético distinto

Durante a fase de acabamento, os bovinos depositam quantidades apreciáveis de gordura subcutânea e visceral, o que prejudica o desempenho produtivo e tem um impacto significativo na qualidade da carne. A composição da gordura intramuscular das carnes de ruminantes influencia a qualidade do produto final, o que explica aimportância de determinar o perfil em ácidos gordos da carne usando diferentes raças e estratégias alimentares. Por outro lado, os ácidos gordos provenientes da dieta, para além de serem biohidrogenados no rúmen, são metabolizados em vários tecidos envolvidos no metabolism lipídico, nomeadamente o tecido hepático. Deste modo, tanto o fundo genético como o sistema de acabamento podem alterar a deposição lipídica, sugerindo assim a sua influência sobre os factores adipogénicos e lipogénicos. O presente estudo visou determinar o efeito da raça e da dieta na composição da carcaça, na celularidade de dois depósitos de tecido adiposo (subcutâneo e mesentérico), bem como as suas implicações no perfil de ácidos gordos e nos níveis de expressão génica de fatores de transcrição e enzimas do metabolismo lipídico. Foram usados quarenta bovinos de duas raças bovinas Portuguesas, a Alentejana e a Barrosã. Os animais foram alimentados com dietas isoenergéticas e isoproteicas, com baixa ou elevada proporção de silagem, até aos 18 meses de idade. Os resultados aqui apresentados sugerem que a raça é o principal determinante da composição da carcaça e qualidade da carne. A localização do depósito de tecido adiposo determinou a area e número dos adipócitos, sendo que no tecido adiposo subcutâneo os adipócitos apresentavam uma área total superior à do tecido adiposo mesentérico. De um modo geral, os resultados demonstram que a composição em ácidos gordos dos depósitos adiposos depende maioritariamente da raça. No entanto, o nível de silagem da dieta teve maior influência no metabolismo lipídico do músculo que no do tecido adiposo subcutâneo, de acordo com os níveis de expressão de genes adipogénicos e lipogénicos. Verifica-se ainda que a dieta influencia a dessaturação e elongação dos ácidos gordos polinsaturados a nível hepático, possivelmente através da expressão de genes que codificam para enzimas associadas a vias de dessaturação/elongação. Estes aspectos salientam que a raça é um factor a ter em conta ao delinear estratégias nutricionais para manipular a partição de tecido adiposo e que têm por base a dieta.

Palavras-Chave: bovinos; composição lipídica; expressão génica; adipogénese; lipogénese; partição de gordura.

List of publications

This PhD thesis was based on the following international peer reviewed papers and manuscripts:

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List of abbreviations

ACACA	Acetyl-CoA carboxylase
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ADG	Average daily gain
Ag^+-HPLC	Silver high performance liquid chromatography
ALP	Alkaline phosphatise (EC 3.1.3.1)
ALT	Alanine aminotransferase (EC 2.6.1.2)
AOAC	Association of Official Analytical Chemists
AST	Aspartate aminotransferase (EC 2.6.1.1)
BCAA	Branched-chain amino acids
BCFA	Branched-chain fatty acids
bp	Base pairs
CIE	Comission Internationale de l'Eclairage
CIISA	Centro Interdisciplinar de Investigação em Sanidade Animal
CLA	Conjugated linoleic acid
CoA	Coenzyme A
CPT1A	Carnitine palmitoyltransferase 1A
CPT1B	Carnitine palmitoyltransferase I B
CRAT	Carnitine acetyltransferase
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid, 22:6*n*-3
DM	Dry matter
DMI	Dry matter intake
DNA	Desoxiribonucleic acid
EC	Enzyme Commission number
ELOVL2	Fatty acid elongase 2
ELOVL5	Fatty acid elongase 5
e.g.	*exempli gratia*
EPA	Eicosapentaenoic acid, 20:5*n*-3
FABP4	Fatty acid binding protein 4
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl ester
FAO/WHO	Food and Agriculture Organization/World Health Organization
FASN	Fatty acid synthase
FMV	Faculdade de Medicina Veterinária
GC	Gas chromatography
HCW	Hot carcass weight
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography

HS	High silage
IDRHa	Instituto do Desenvolvimento Rural e Hidráulica
i.e.	*id est*
IL-6	Interleukine-6
IMF	Intramuscular fat
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
INSR	Insulin receptor
LA	Linoleic acid, 18:2*n*-6
LC	Liquid chromatography
LCFA	Long chain fatty acids
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL	Low density lipoprotein
L-INIA	Laboratório de Investigação Agrária
LL	*Longissimus lumborum*
LPL	Lipoprotein lipase
LS	Low silage
LT	*Longissimus thoracis*
MAT	Mesenteric adipose tissue
mRNA	messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
NCBI	National Center for Biotechnology Information
NL	Neutral lipids
ns	Not significant
OBCFA	Odd- and branched-chain fatty acids
OCFA	Odd-chain fatty acids
OD	Optical density
P	Probability
PCA	Principal Component Analysis
PDO	Protected Designation of Origin
pH	Potential of hydrogen
PL	Polar lipids
PPARA/PPARα	Peroxisome proliferator activated receptor alpha
PPARG/PPARγ	Peroxisome proliferator activated receptor gamma
PPIB	Peptidylprolyl isomerase B
PUFA	Polyunsaturated fatty acids
RPLP0	Ribosomal protein, large, P0
RT-qPCR	Real time quantitative polymerase chain reaction
SAT	subcutaneous adipose tissue
SAS	Statistical analysis system
SCD	Stearoyl-CoA desaturase
SDHA	Succinate dehydrogenase complex subunit A
SE	Standard error
SEM	Standard error of the mean

SFA	Saturated fatty acids
SREBF1	Sterol regulatory element binding factor 1
SREBP	Sterol regulatory element binding protein
ST	*Semitendinosus*
SW	Slaughter weight
TAG	Triacylglycerols
TFA	*Trans* fatty acids
UFA	Unsaturated fatty acids
VFA	Volatile fatty acids
vs.	*versus*
WBSF	Warner-Bratzler shear force

List of symbols and units

%	Percent
±	Standard deviation/Standard error/Standard error of the mean
<	Less than
>	Greater than
°C	Degree Celsius
c	*cis*
g	Gram
h	Hour
hPa	Hectopascal
kg	Kilogram
M	Molar
mg	Miligram
mL	Mililitre
μg	Microgram
μL	Microlitre
μm	Micrometre
min	Minute
mm	Milimetre
n	Number of experimental units
ng	Nanogram
nm	Nanometre
r	Correlation coefficient
rpm	Revolutions per minute
t	*trans*

INTRODUCTION

Ruminant meat, milk and dairy products are important lipid sources for human diets. However, the increased production and consumption of ruminant edible products, rich in saturated (SFA) and *trans* fatty acids (TFA) on one side, and the nutritional guidelines from FAO/WHO advising for its replacement by polyunsaturated fatty acids (PUFA) rich foods, on the other side, pose an important challenge to the beef industry. Ruminant edible products should therefore be leaner and have their content in beneficial fatty acids, such as *n*-3 PUFA, increased. To approach this objective, insights on the complexity of ruminants' fatty acid metabolism and pathways used for lipid deposition are needed.

Adipose tissue, besides being a component of the bovine carcass composition, is also a determinant of carcass quality and economic value. With the growing interest of beef industry in providing a healthier product, it became apparent that different feeding strategies can lead to changes in the quantity and profile of lipids deposited in beef. Fat accretion in beef cattle is, therefore, of great practical importance. Deposition of fat is less efficient than that of lean, which reflects very poor feed conversions of cattle nearing slaughter. Excessive fat trim (*i.e.* internal/visceral, intermuscular and subcutaneous fat) is penalised. One of the most important issues in the animal industry is the production of high-quality meat at low cost, and a better understanding of the specific accumulation mechanisms of fat depots will contribute to improve production efficiency. Thus, there are conflicting interests for the production of fat, with intramuscular fat commanding a price premium (and regarded as "taste fat") and other depots incurring penalties (and considered as "waste fats"). In view of the mentioned above, further studies of the genetic, nutritional and physiological factors affecting fat distribution are necessary.

Attending that feed utilization and partitioning of nutrients to various tissues is influenced by the visceral organs, it is important to understand their dynamics in order to explain the ultimate body composition of the animal. Fat, being the most variable tissue in the carcass, has a large influence on the proportions of bone and muscle (Berg & Butterfield, 1976). However, within the adipose tissue, fat depots are affected differently by diet composition. Therefore, the manipulation of adipose tissue metabolism may support better performance as well as improved quality of meat products (Hocquette, Ortigues-Marty & Vermorel, 2001). Currently, the challenge is to manipulate lipid metabolism of tissues in order to optimize the muscle metabolic features which determine meat fatty acid composition.

The beef industry has sought to optimize their cattle management strategies to produce beef with increased monounsaturated fatty acids (MUFA) and decreased SFA contents, thus providing healthier products for human consumption. In most industrialized countries, a high

meat intake contributes to higher than recommended total fat (15-30% of calories) and saturated fatty acids (<10% of calories) intakes, which are associated with an increased risk of obesity, hypercholesterolemia and some cancer types (Wood *et al.*, 2008). Meat accounts for 10-20% of the total calories in human diets and ruminant meats are high in SFA (up to 50%) (Chizzolini *et al.*, 1999). In addition, it is well known that the lower polyunsaturated fatty acids to SFA (PUFA/SFA) and higher *n*-6/*n*-3 ratios in some meats contribute for the imbalance in the fatty acid intake of today's consumers (Wood *et al.*, 2008). Furthermore, typical western diets display low ratios of PUFA/SFA, which have been considered as major risk factors for cardiovascular diseases, and high *n*-6/*n*-3 ratios (15-17/1), which favour the development of cardiovascular diseases, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2002). Consequently, during the last decade, a considerable research effort has been made in order to improve production efficiency, fat partition and deposition, as well as the nutritional value of ruminant edible products.

White adipose tissue expands by hyperplasia (cell proliferation) or hypertrophy (cell, enlargement) (Gregoire, 2001). Thus, differentially expressed genes that regulate cell proliferation and differentiation in adipose tissue may play a role in adipogenesis. For instance, animals with high backfat thickness have more ongoing adipogenesis than those with low backfat thickness (Jin *et al.*, 2012). Understanding the genetic mechanisms influencing differences in fatty acid composition, *e.g.* desaturase and elongase activities, could lead to the development of novel genetic programmes directed to the improvement of meat quality. Furthermore, the genetic profiles of Portuguese autochthonous purebred bovines have been described, with Alentejana purebred being phylogenetically distant from Barrosã purebred (Beja-Pereira *et al.*, 2003). The contribution of the genetic factors to the differences obtained in fatty acid composition of beef lipids remain to be studied.

In view of the above, it is of paramount importance to gain insight on the genetic, metabolic and endocrine regulation of fat deposition and fatty acid metabolism in diverse bovine breeds. Moreover, it is necessary to fully characterize and understand the effects of diet composition to devise breed-specific strategies to manipulate fatty acid composition of ruminants' edible products.

This thesis comprehends 7 chapters. Chapter 1 ("Scientific background and objectives") revises our current knowledge on ruminant lipid metabolism, as well as on the genetic and environmental factors that influence adipogenesis and lipogenesis in ruminants. The first section concludes with a description of the objectives of this work. Chapters 2 to 7 are based on scientific manuscripts, which were accepted or submitted for publication in international peer reviewed journals. Each chapter comprises an abstract, introduction, description of experimental procedures, results, discussion and conclusions.

Chapter 2 (“Productive traits and carcass composition”) explores the effect of breed and dietary silage on meat quality and carcass fat partitioning in Alentejana and Barrosã young bulls. Chapter 3 (“Cellularity and lipid profile of subcutaneous and mesenteric adipose tissues”) describes the detailed fatty acid composition and cellularity of two major, and distinct, fat depots: mesenteric and subcutaneous adipose tissues. Chapter 4 (“Meat lipid profile”) analyses the effects of breed and diet composition on the fatty acid composition and gene expression in the *longissimus lumborum* (LL) muscle and subcutaneous adipose tissue. Chapter 5 (“Gene regulation of fatty acid composition in subcutaneous adipose tissue and muscle”) investigates the effect of breed and dietary silage on meat fatty acid composition from a biochemical standpoint. This chapter is focused on a detailed analysis of fatty acid composition of total, neutral and polar lipid fractions in the LL muscle. Chapter 6, entitled “Hepatic regulation of lipid metabolism”, addresses the role of liver in the overall lipid metabolism of ruminants, with a particular emphasis on the pathways that lead to the synthesis of *n*-3 long chain PUFA. This study by establishing a direct comparison, in terms of fatty acid composition, of the hepatic tissue and other tissues involved in lipid metabolism: muscle, and subcutaneous and mesenteric adipose tissues.

These last two chapters of the thesis, chapters 7 (“General discussion”) and 8 (“General conclusion, implications and future perspectives”), aim to discuss in an integrated way the results obtained in each of the five previous chapters, allowing to derive the main general conclusions of this project and to describe the relevant perspectives for future research on this field.

CHAPTER 1

Scientific background and objectives

1.1 Beef cattle production systems

Beef cattle production systems can be extensive, semi-intensive or intensive. Extensive systems are grazing-based, whereas in intensive systems animals are kept indoors and fed high concentrate diets with just enough straw to maintain rumen function. In the semi-intensive production systems animals are raised according to any combination of both extensive and intensive husbandry methods, according to changes in climatic conditions or physiological state.

Intensive livestock production systems aim to maximize the genetic potential of animal breeds and feeds. Genotypes used in these production systems usually display high growth rates and feed efficiencies. Intensive systems use cultivated crops or harvested feeds, or both, for most part the production system, but in particular in the finishing period. Animals are provided diets based on concentrates, in which cereals and grains predominate and, therefore, present a high density of nutrients, with high digestibility, low fibre and high energy contents. In intensive systems, and especially during fattening, concentrates may make up to 70% of the diet. Ruminant production systems of lower intensity may sometimes use concentrate feeds to supplement forage-based diets. Thus, concentrate feeding improves livestock productivity which is generally associated with an improved biological efficiency of feed use while promoting a better utilization of roughage feeds for ruminant livestock.

Grass-based beef production systems are low-input systems that are particularly suitable to meet the demand of meat retailers and consumers for naturally and animal-friendly produced beef (Razminowicz, Kreuzer & Scheeder 2006). Moreover, the perceived healthiness of food and the protection of environmental quality has become a key quality issue for consumers (Rodrigues, Andrade & Rodrigues, 1998). The extensive livestock production systems are more “natural” and it is accepted that they tend to have advantages over intensive systems in terms of ecological sustainability (Rodrigues *et al.*, 1998). Beef cattle production from indigenous breeds allows producing quality products and allows for a more balanced use of natural resources (Table 1.1). The traditional Portuguese extensive production systems are sustainable and based on the use of local available resources – indigenous cattle breeds and natural or seeded forages (Rodrigues *et al.*, 1998). Still, investigation on the productive parameters of the autochthonous breeds is necessary to improve the production performance under specific production conditions, and to assure the preservation of the characteristics its products.

In Portugal there are two main extensive production systems - one in the North and Center of the country, and another one in the South. Both systems differ in the edapho-climatic conditions, agrarian structure, type of supplementary feeding, calving period and slaughter age. In the South there are two calving periods (Summer and Winter) and animals are

slaughtered at 18 to 24 months of age, whereas in the North calving is continuous and animals are 7 to 9 months old at slaughter (Rodrigues *et al.*, 1998).

Table 1.1 – Some differential characteristics of the extensive and intensive production systems. Adapted from Rodrigues *et al.* (1998).

	Extensive	**Intensive**
Genetic resources	indigenous breeds	mainly crossbred imported×indigenous breeds
Feed	local resources indigenous flora quality variable with annual cycle produced to local capacity of land	mainly imported concentrate high energy more expensive
Produce quantity and quality	limited supply specific characteristics	mass supply standardised product
Demand	niche market	wide market
Cost per unit produced	more expensive	less expensive
Stocking density	low aims to optimise use of locally available forage resources	high energy aims to maximize income
Animal - Biological adaptation	well adapted, but less productive	higher sensitivity to production diseases higher production capacity
Location	regional	general
Conservation of genetic resources	positive	potentially adverse

A significant percentage of extensive cattle production is based on the use of animals with unique characteristics, good adaptation to the environment, robustness but modest growth performance. There are 13 Portuguese native cattle breeds with protected names (Instituto do Desenvolvimento Rural e Hidráulica, IDRHa, 2007), raised in specific geographic regions, which have distinct morphological features. Several studies have reported high within-breed genetic diversity for the Portuguese breeds (Mateus, Penedo, Alves, Ramos & Rangel-Figueiredo, 2004; Ginja, Gama & Penedo, 2010). In addition, and despite the small geographic area where these cattle are raised, there are low levels of gene flow among breeds which are therefore highly structured (Ginja *et al.*, 2010), as illustrated by Figure 1.1.

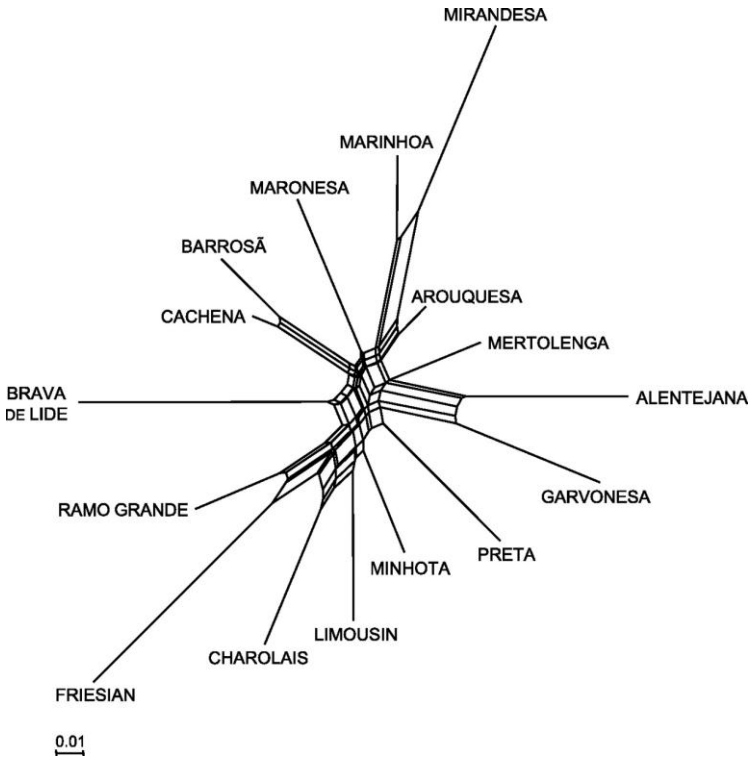

Figure 1.1 – Genetic distances among 13 Portuguese native and 3 imported cattle breeds. Adapted from Ginja *et al.* (2010).

Differences in lipid composition and nutritional quality of intramuscular fat of four Portuguese autochthonous breeds have been described (Alfaia *et al.*, 2006a; Alfaia *et al.*, 2006b; Alfaia *et al.*, 2007a; Alfaia *et al.*, 2007b). Furthermore, specific genetic characteristics have been described for Portuguese autochthonous bovine breeds, with Alentejana being phylogenetically distant from the Barrosã breed (Beja-Pereira *et al.*, 2003; Ginja *et al.*, 2010). In addition, Alentejana and Barrosã originate from quite distinct geographic regions and are raised under different systems. Alentejana is a large-framed breed usually produced in a traditional semi-intensive production system in Alentejo and some councils of the Setúbal district (south of Portugal). This breed is generally produced in an extensive grazing system based on natural pastures of Montado with a finishing period on concentrate during 3 to 6 months (Alfaia *et al.*, 2006a). The Barrosã is a small-framed, long horned breed from the mountainous Northwest of Portugal (Highlands of Minho and Terras do Barroso), still used for tilling the small plots that characterise land ownership in the Northern part of the country (Silva, Lemos, Monteiro & Vaz Portugal, 1998). Generally, Barrosã purebred calves are produced in a traditional production pasture-based system (Alfaia *et al.*, 2007a).

1.2 Carcass fat partitioning

Differences at a cellular, metabolic and genetic level among adipose depots have been reported in meat animals (Hausman *et al.*, 2009; Dodson *et al.*, 2010a; Dodson *et al.*, 2010b). There are a number of intrinsic and extrinsic characteristics that are known to influence the expression of marbling, though this may not result specifically from hyperplastic growth from pluripotent stem cells. Age is a normal determinant of adiposity in mammals, with the developmental programmes of growth, puberty and ageing driving significant changes in fat distribution and total fatness (Vernon, 1981; Vernon, Finley, Taylor & Flint 1985; Kirkland, Tchkonia, Pirtskhalava, Han & Karagiannides, 2002).

Organs and tissues do not mature simultaneously. In fact, a general gradient in the formation of tissues and organs from head to tail and from extremities to the core is usually observed (Owens, Dubeski & Hanson, 1993). Consequently, as growth proceeds, a shift occurs in lipid deposition is diverted from the peripheral fat depots to the intramuscular adipose tissue. In addition, Carter *et al.* (2002) suggested that intramuscular fat deposition is accelerated once animals reached a particular body weight. Increased marbling has also been negatively associated with concentrations of stearic and linoleic acids, as well as PUFA (Xie *et al.*, 1996).

Regional differences also exist in developmental morphology, cellularity, lipid content, responsiveness to metabolic regulators, and the ability to mobilize lipids in animal and tissue-specific adipose depots (Coppack, 2005; Spalding *et al.*, 2008). Moreover, adipogenesis (Dodson *et al.*, 2010a; Fernyhough *et al.*, 2005) inside skeletal muscle during the foetal and early postnatal stages has a dominant effect on the number of postnatal intramuscular adipocytes (Dodson *et al.*, 2010a; Du *et al.*, 2010), because the total number of adipocytes appears to be set when reaching adolescence in some (but not all) meat animals (Spalding *et al.*, 2008). However, the mechanisms that control adipogenesis in the foetal and postnatal stages in all of the adipose depots of meat animals remain poorly defined (Dodson *et al.*, 2010a).

Polar lipid fatty acids in intramuscular fat of the LL muscle contain a higher proportion of polyunsaturated fatty acids and a lower proportion of saturated fatty acids compared to the TAG fatty acid fraction, which may reflect a prerequisite for proper membrane functioning (Webb, De Smet, Van Nevel, Martens, & Demeyer, 1998). Internal fat depots are more saturated compared to subcutaneous fat. The proportion of monounsaturated fatty acids differ between intramuscular fat in *transversalis* and in *serratus* muscles, possibly reflecting differences in muscle activity and functioning.

1.2.1 Role of fat depots in lipid metabolism

Although adipose tissue has higher fatty acid content than muscle, the fatty acid proportions of the two tissues are similar (Enser, Hallett, Hewitt, Fursey & Wood, 1996). Oleic and stearic acids comprise more than 60% of the total fatty acids in all anatomical locations of livestock (Webb *et al.*, 1998) and occur predominantly in the TAG fraction.

The fat depot location determines its metabolism. Intramuscular fat deposition is apparently regulated by factors that are different than those regulating fat deposition in other tissue depots, while there are metabolic differences between intramuscular and subcutaneous fat depots (Miller, Cross, Lunt & Smith, 1991; Kokta, Dodson, Gertler & Hill, 2004). Miller *et al.* (1991) and May *et al.* (1994) reported that in cattle, intramuscular adipocytes are smaller than subcutaneous fat cells. Similar results were reported by Eguinoa *et al.* (2003). Moreover, the activity of some glycolytic enzymes is higher in intramuscular depots. In contrast, subcutaneous fat depots display higher levels of lipogenic enzymes, thus suggesting that each depot has unique aspects related to lipid metabolism. Furthermore, breed has also been pointed as a factor influencing the lipogenic activity of each depot (Miller *et al.*, 1991; May *et al.*, 1994).

Intermuscular fat depots have similar characteristics to intramuscular fat depots. Eguinoa *et al.* (2003) reported that in cattle, the intermuscular fat depot had the smallest adipocyte size when compared to omental, perirenal, and subcutaneous depots. Additionally, the intermuscular depot additionally had a lower level of lipogenic enzyme activity when compared with other depots, such as the intramuscular fat depot. However, when size was adjusted, subcutaneous and intermuscular fat depots had higher enzyme activity than other depots, indicating a potential role of other factors such as blood flow and lipolytic activity as determinants for the depot differences observed (Eguinoa *et al.*, 2003). There appears to be higher leptin mRNA levels in subcutaneous than omental fat depots, but this may be due to adipocyte volume. It has been reported that leptin is correlated to adipocyte volume, and subcutaneous adipocytes are larger than omental adipocytes (Zhang *et al.*, 2002).

Studies by Smith and Crouse (1984) have shown that adipocytes associated with the intramuscular fat depot have a higher reliance on glucose and/or lactate as a substrate than acetate. Intramuscular adipocytes have lower rates of lipogenesis and associated smaller cell size than other depots (Vernon, 1981) but show an increased absolute and relative reliance on glucose for lipogenesis.

The subcutaneous adipose depot is one of the largest adipose depot and, in beef animals, this depot displays different regulatory mechanisms when compared with other adipose depots (Romão *et al.*, 2011). Moreover, different locations of the type of adipose tissue may show distinct metabolic profiles, as suggested by Romão *et al.* (2011) who found different

profiles of microRNA expression in abdominal, back and rump subcutaneous adipose tissues.

1.2.2 Depot effects on fat interaction with muscle

The location of the adipose tissue depots impacts on adipocyte proliferation, differentiation, metabolism and ways of interaction with the muscle tissues (Kokta *et al.*, 2004). The proximity of the adipose tissue to muscle may explain some of these effects. However, some internal depots - omental, mesenteric, and perirenal - are not close to muscle, implying that their influence is exerted through endocrine mechanisms (Kokta *et al.*, 2004). On the other hand, in adipose tissue depots such as subcutaneous, intermuscular, or intramuscular, signalling may occur *via* endocrine or paracrine mechanisms (Kokta *et al.*, 2004). Furthermore, considering that proteins with endocrine function are also secreted by the skeletal muscle tissue, the spectrum of potential direct communication between skeletal muscle and adipose tissue is considerably broadened (Komolka, Albrecht, Wimmers, Michal & Maak, 2013). Gondret, Guitton, Guillerm-Regost & Louveau (2008) detected almost 150 proteins considered as characteristic for intramuscular fat (IMF). It is possible that IMF influences the metabolism of surrounding muscle cells and a reciprocal influence of myocytes on intramuscular adipocytes might exist (Komolka *et al*., 2013). Hence, depot location may play a significant role in the physiological interactions of adipose tissue.

1.3 Lipid composition of ruminant fats

Lipid deposition, particularly of SFA and conjugated linoleic acid (CLA), in muscle tissues occurs mainly in the neutral lipids (NL) fraction, whereas the PUFA are preferentially deposited in the polar lipids (PL) fraction (Lourenço, 2007). In ruminant muscles, it is know that CLA is mainly associated with the TAG fraction, which is related to the fat content of tissues (Raes, De Smet, Balcaen, Claeys & Demeyer, 2003). Ruminants tend to incorporate essential fatty acids preferentially into muscle lipids, especially phospholipids, rather than storing them in the adipose tissue (Wood *et al.*, 2008).

The saturation of fats is a direct consequence of ruminal biohydrogenation of unsaturated fatty acids by ruminal microorganisms. Thus, improvement of fatty acid profiles of ruminant products may be achieved by two distinct approaches: either by the modification of the fatty acid profile during meat or milk processing or by the modification through the changes in

animal diet. The latter might simply result in greater bypass of dietary fatty acids from the rumen, or might be a consequence of altered microbial metabolic activity.

1.3.1 Factors influencing fat content and fatty acid composition of ruminant fat depots

The degree of fatness is a major factor influencing fatty acid composition of ruminant fat depots. In turn, fatness is determined by three major factors: genetic background, diet composition and age.

1.3.1.1 Genetic background

Breed and genetics have an important role in fat accretion. Wagyu and Belgian-Blue breeds are two opposite examples regarding intramuscular fat development. Studies with reference to the effect of age or live weight on intramuscular fat composition have also been related to body fatness. Nürnberg, Wegner & Ender (1998) showed that with increasing age/weight, animals gain a tendency to deposit higher amounts of fat leading to an increase in SFA and a decrease in PUFA levels. In fact, it is accepted that the proportion of PUFA increases with decreasing level of fatness related to higher proportions of phospholipids (Indurain *et al.*, 2006).

The breed and genotype are among the main factors affecting fatty acid composition, given the differences in fat deposition between breeds and its relation to the ratio of TAG to phospholipids (Raes, De Smet & Demeyer, 2001; Aldai *et al.*, 2006). Even though breed differences are generally small, they reflect differences in fatty acid metabolism, gene expression or enzyme activity, and, therefore, deserve some further consideration (Scollan *et al.*, 2006; Bartoň, Marounek, Kudrna, Bureš, & Zahrádková, 2007). In fact, Δ^9, Δ^6 and Δ^5 desaturases, elongase, SREBP-1c and leptin have been regarded as the most important genetic factors associated with fatty acid deposition in carcass fat. Expression and activity levels of the Δ^9 desaturase enzyme are associated with MUFA content in the muscle of ruminants as well as the levels of CLA, as it converts the 18:1*t*11 into *c*9,*t*11, the major CLA isomer in ruminant fats (Lourenço, 2007). Recently, Warren *et al.* (2008) showed that Holstein-Friesians steers fed grass silage formed more docosahexaenoic acid (DHA) in phospholipids from its precursor supply (18:3*n*-3) than Aberdeen Angus steers, concluding that the activity and/or an expression of Δ^5 and Δ^6 desaturase enzymes is higher in Holstein–Friesians. The activity of both enzymes, as well as the precursor supply, will determine the

extent of these processes and the outcome proportions of long chain PUFA (LC-PUFA) in intramuscular fat (De Smet, Raes & Demeyer, 2004; Scollan *et al.*, 2006).

1.3.1.2 Diet composition

Diet is known to impact many phenotypic traits in ruminants such as growth rate, and meat and milk fat content and composition (Dian, Andueza, Jestin, Prado & Prache, 2008; Romão, Jin, He, MsAllister & Guan, 2012). Therefore, beef cattle producers can, by manipulating the diet and feedstuffs, obtain meats that more closely meet the expectations of consumers, regarding its organoleptic and nutritional quality.

Altering the IMF fatty acid composition of ruminants through diet composition poses a greater challenge when compared to monogastric animals (Demeyer & Doreau, 1999; Rhee, 2000). Ruminant diets are usually low in fat but high in PUFA, whether it is fresh or conserved forage. However, a high proportion of PUFA undergo microbial biohydrogenation and therefore the fatty acids absorbed in the small intestine and deposited in tissues are predominantly SFA. Therefore, the major limitation to manipulating fatty acid composition of ruminant products through dietary means is microbial biohydrogenation of dietary unsaturated fatty acids in the rumen (Figure 1.2), which is largely responsible for the high saturated nature of ruminant products (Lee, Tweed, Dewhurst, & Scollan, 2006).

Beef cattle feeding systems, whether using concentrate, pasture feeding or both, influence the growth and carcass characteristics of animals. While attempting to improve fatty acid composition, the growth parameters may be influenced negatively. The ideal growth performance and ideal fatty acid composition are often negatively correlated under natural feeding conditions. The meat from ruminants reared under pasture-based systems differs from those of grain-fed animals in flavour, tenderness, colour, as well as lipid content and composition (Realini, Duckett, Brito, Rizza & De Mattos, 2004; Descalzo *et al.*, 2005). Indeed, several factors have been identified as affecting the fatty acid content and composition of ruminants, notably forage:concentrate ratio of the diet (Santos-Silva *et al.*, 2002; Lee *et al.*, 2006).

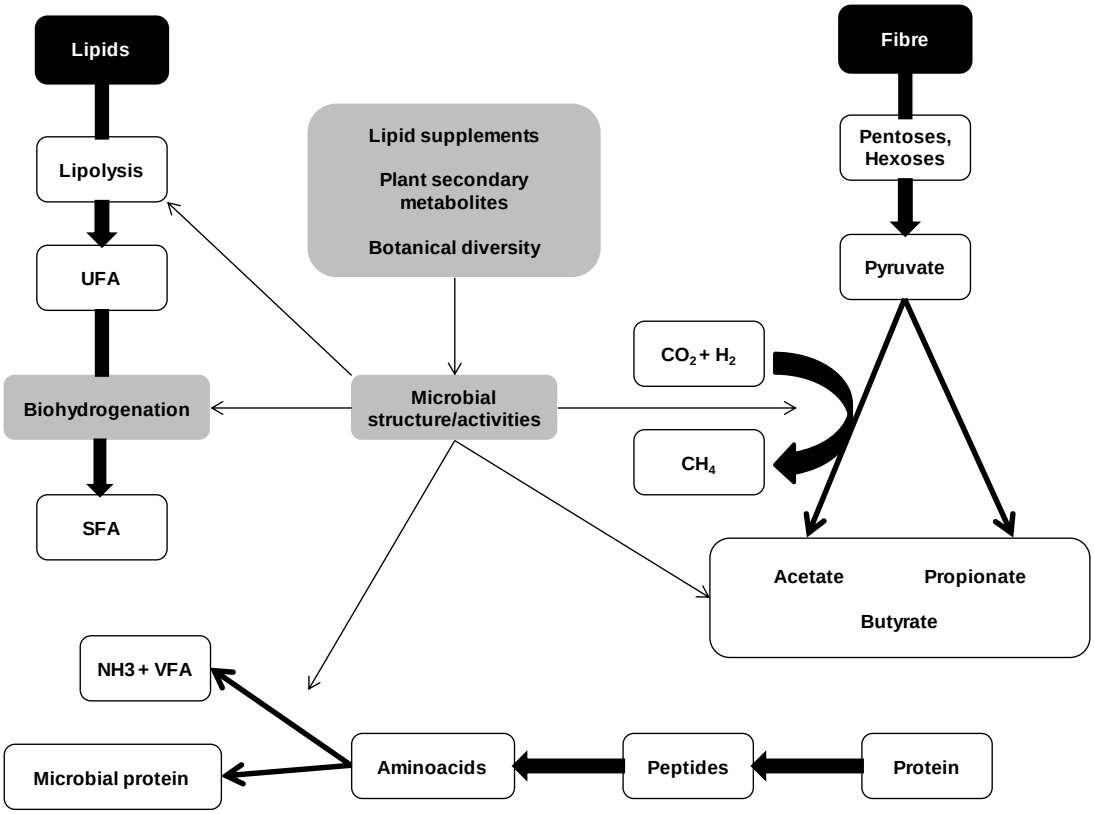

Figure 1.2 – Possible interventions to manipulate lipid metabolism in the rumen and implications on other processes (SFA, saturated fatty acids; UFA, unsaturated fatty acids; VFA, volatile fatty acids). Adapted from Lourenço *et al*, (2010).

One major nutritional strategy to promote IMF deposition is to increase the availability of net energy, and therefore glucose, for fat synthesis during the finishing period (Harper & Pethick, 2004). However, as maturing proceeds the deposition of SFA in the adipose tissues increases, so the outcome of promoting carcass fat deposition during the finishing phase is a lowered PUFA/SFA ratio (Wood *et al.*, 2008). Still, the amount of fat and PUFA/SFA ratio may be improved by feeding animals to a predetermined degree of fatness. In addition, there are positive aspects of feeding high-grain diets during the finishing period, namely improved animal performance and shortening of the finishing periods.

In many species, the concentration of oleic acid in adipose tissue reflects the average concentration of oleic acid in the diet (St John *et al.*, 1987), but in ruminants oleic acid is hydrogenated to stearic acid by ruminal microorganisms (Ekeren, Smith, Lunt & Smith, 1992). The fatty acid pattern of ruminant fat depots is very complex and is mostly determined by the interaction between dietary factors and rumen metabolism (Alves & Bessa, 2007). In fact, the ruminal microbial activity largely influences the pattern of fatty acids available to the animal. Rumen biohydrogenation of dietary PUFA is modulated by several factors, resulting

in differences in the amount of PUFA that escapes biohydrogenation and in the type of biohydrogenation intermediates (Bessa *et al.*, 2007).

Beef from pasture-fed ruminants contains increased concentrations of *n*-3 PUFA and CLA, and decreased SFA compared to meats from concentrate-fed animals (Scollan *et al.*, 2006). In contrast, diets rich in grains and protein supplements have higher amounts of 18:2*n*-6, the precursor of the *n*-6 PUFA. The higher PUFA percentage in meat from pasture-fed cattle may be due to higher protection of fatty acids from ruminal biohydrogenation in fresh grass, relative to grain or silages (Eriksson & Pickova, 2007).

The diet composition also modulates the content and proportions of the individual CLA isomers of ruminant tissues (Mir *et al.*, 2004; Nuernberg *et al.*, 2005). Finishing ruminants on pasture has been shown to promote total CLA deposition (Santos-Silva, Bessa & Santos-Silva, 2002; Nuernberg *et al.*, 2005) because the growth of fibrolytic microorganisms responsible for the rumen production of CLA is increased (Duckett & Gillis, 2010). In contrast, concentrate-fed beef cattle have lower CLA contents due to lowered rumen pH and PUFA content, when compared to fresh pastures (van Soest, 1994; Bessa, Santos-Silva, Ribeiro & Portugal, 2000).

Ruminal biohydrogenation may be reduced by increasing of the concentrate proportion in the diet. Typically, corn-based diets have been shown to have high percentages of palmitic, oleic and linoleic acids. These diets also contain low levels of stearic and palmitoleic acids (Ducket, Andrae & Owens, 2002). Concentrate diets may defaunate the rumen because they lower the ruminal pH (Hook, Steele, Northwood, Wright & McBride, 2011). Protozoal defaunation is also caused by long-chain unsaturated fatty acids and medium-chain saturated acids, which are found in cattle fed high-grain diets (Hristov, Ivan & MacAllister, 2004; Hristov, Callaway, Lee & Dowd, 2012). Carbohydrates, such as starch, are rapidly fermented by ruminal microorganisms, often resulting in a decline in ruminal pH (Annison & Bryde, 1998). This response appears to be associated with microbial changes in rumen bacteria, reduced cellulolytic bacteria, including *Butyrivibrio fibrisolvens,* which is known to produce the precursor of *c*9,*t*11 in ruminant tissues, 18:1*t*11. The higher microbial population of lactate-producing (*Streptococus bovis*) and lactate-utilizing bacteria (*Selenomonas ruminantium* and *Megasphera elsdenii*) leads to the formation of 18:1*t*10 and *t*10,*c*12 CLA (Hudson, Mackenzie, & Joblin, 1995; Lee *et al.*, 2006). According to Griinari & Bauman (1999) decreasing the forage proportion in the diet increases the duodenal flow of 18:1*t*11 and results in a shift in the ruminal production of CLA from *c*9,*t*11 CLA towards *t*10,*c*12 and its reduction to 18:1*t*10.

Dietary fat changes alter gene expression in bovine adipose tissue (Joseph *et al.*, 2010; Romão *et al.*, 2012). For instance, the increase in MUFA contents in the subcutaneous adipose tissue as fattening proceeds is due to an increase in stearoyl-CoA desaturase (SCD)

gene expression and a concomitant catalytic activity (Chung, Lunt, Kawachi, Yano & Smith, 2007; Jiang *et al.*, 2008; Duckett, Pratt, & Pavan, 2009). In contrast, pasture or hay feeding is reported to have an inhibitory effect on SCD gene expression (Ducket *et al.*, 2009; Smith *et al*., 2009a), with a concomitant increase in SFA contents. Increased dietary PUFA may limit ruminal production of CLA and vaccenic acid and/or depress SCD expression or activity in lean tissues, thus limiting CLA formation and accretion in tissues (Daniel, Wynn, Salter & Buttery, 2004).

1.3.1.3 Age

The age specifically affects the MUFA contents, through *SCD* gene expression and enzyme activity (Smith *et al.*, 2009a). Usually, the MUFA:SFA ratio increases with age, in neutral lipids of muscle and total adipose tissue of beef cattle (Wood *et al.*, 2008; Smith *et al.*, 2009b). The increasing importance of neutral lipids in total lipids as fattening proceeds and the fairly constant level of phospholipids were addressed by Warren *et al.* (2008). The authors reported that the proportion of phospholipids in total lipids fell from 30% at 14 months to 12% at 24 months and this was accompanied by an increase in the proportion of 18:1*c*9 and a decrease in the proportion of 18:2*n*-6 in total lipids.

1.4 Meat quality

Carcass-related parameters and the factors that influence the accumulation, distribution and composition of carcass fat in beef cattle have been, for many years, the subject of extensive research. However, the role, value and perception of the importance of animal fat depots in meat quality differ considerably between producers, abattoirs, butchers, retailers and consumers. Fat and fatty acids, regardless of fat depot, contribute to important aspects of meat quality and are central to the nutritional and sensory values of meat.

1.4.1 The role of adipose tissue

Depending on the fat depot location, the adipose tissue may have an impact on meat quality related parameters in different ways. Subcutaneous fat contributes to protect the carcass from cold shortening during the chilling process (Dolezal, Smith, Savell & Carpenter, 1982). It should be highlighted that the increase in intramuscular fat results from a general carcass fat

increase and, therefore, the effects of breed and diet on the IMF accretion should be framed on a larger context. In addition, meat flavour, juiciness, tenderness benefits from IMF (Wood *et al.*, 2008).

1.4.2 pH

In vivo, normal pH in skeletal muscle is approximately 7.2. At 24 to 48 hours *post-mortem* the skeletal muscle pH is about 5.4-5.7. The pH drop rate (glycolytic rate) and the final pH (glycolytic potential) are major influencing factors on meat quality (Purchas, Yan & Hartley, 1999). A moderate rate of pH decline, that is when a pH between 6.2 and 6.7 is reached in the first 3 hours *post-mortem*, has beneficial effects on beef tenderness (Klont *et al.*, 2000). Simmons *et al.*, (2008) suggested that pH may influence the skeletal muscle cell proteolytic enzymes.

1.4.3 Colour

Colour is an important determinant of the visual appearance of meat and thus influences consumer's choices. Meat colour is related to the ultimate meat pH, which in turn, is associated with tenderness (Purchas, 1990). In the Comission Internationale de l'Eclairage (CIE) L* a* b* system, the L* component refers to brightness (value), whereas a* and b* are the chromaticity coordinates. L* values vary between 0 (dark) and 100 (light). The coordinate a* (redness) measures the ratio between green (-60) and red (+60) and the coordinate b* measures the ratio between blue (-60) and yellow (+60). These coordinates can also be used to calculate the hue angle (h*) and chroma (C*).

The hue angle, commonly designated by colour, is mostly determined by pigment content and chemistry (Renerre, 2000) and indicates the wavelength of reflected light (Sahin & Sumnu, 2006). Chroma indicates the degree of deviation from an established neutral value of grey (Sahin & Sumnu, 2006) and depends mostly on the myofibrillar structure and on the ultimate meat pH (Renerre, 2000).

1.4.4 Cooking loss and shear force

Cooking loss, that is the difference between the weight before and after cooking, is influenced by factors such as the IMF and moisture contents. Generally, meats with high IMF

content sustain lower thaw-drip and total cooking loss, whereas meats higher in moisture sustain greater thaw-drip and total cooking loss (Jeremiah, Dugan, Aalhus & Gibson, 2003). When the influence of diet composition on cooking loss was investigated, Varela *et al.* (2004) observed no differences in drip loss, water holding capacity and cooking loss between beef from pasture-fed cattle and grain-fed cattle, when animals were compared at similar weights or fat cover.

Tenderness and tenderness-related traits are highly variable within and among beef muscles (Costa *et al.*, 2012), which is due to differences in proteolysis, rigour shortening and/or connective tissue properties (Rhee, Wheler, Shakelford & Koohmaraie, 2004).

1.5 Lipid metabolism in ruminants

1.5.1 Adipogenesis

Adipogenesis, the complex development from preadipocytes or mesenchymal stem cells to mature adipocytes, is essential for fat formation and metabolism of adipose tissues in mammals (Romão *et al.*, 2011), and is accompanied by marked morphological and biochemical changes. Adipogenesis has been one of the most intensely studied models of cellular differentiation. Outside of its role as a model system to help answer general questions in developmental biology, adipose tissue is an important component of the body's system of energy balance (Rosen & Spiegelman, 2000).

Adipose tissue is an important organ that is involved in the peripheral regulation of body homeostasis, specifically, energy intake, storage and expenditure (Roh, Hishikawa, Hong & Sasaki, 2006). The adipocytes play a central role in the regulation of lipid metabolism, acting both in the storage of fatty acids and as an endocrine cell to regulate energy expenditure and feeding behaviour (Roh *et al.*, 2006). The development of adipose tissue is controlled by the balance between cell proliferation (hyperplasia) and size increase (hypertrophy), which is due to the assimilation of fatty acids into TAG-rich lipid droplets. In the adipocyte, hypertrophy mainly involves accumulation of intracellular lipid.

Individual fat depots display different rates and time periods of hyperplastic and hypertrophic growth (Hammond, 1955; Robelin, 1986). Visceral, subcutaneous, intermuscular, and intramuscular fat depots differ in their timing of formation and metabolic role. Visceral fat is developed first to provide neonates insulation and organ protection, whereas intermuscular and subcutaneous fat are often developed simultaneously. Intermuscular fat is the largest depot in a beef carcass, followed by subcutaneous fat. Intramuscular fat is the last depot to

develop, which is why maturity heavily regulates the development of this fat depot (Hammond, 1955; Hood & Allen, 1973).

Lipid deposition, especially in subcutaneous adipose tissues, is directly associated with the yield and the quality of meat (Powell & Huffman, 1973). Marbling is highly regulated by growth. Cianzio, Topel, Whitehurst, Beitz and Self (1985) found that, between 11 and 19 months, subcutaneous, intermuscular, kidney and mesenteric fat depots in steers grew mostly by hypertrophy. The intramuscular fat growth, on the other hand, was due to the development of new adipocytes as well the increase of their lipid content.

1.5.2 Lipogenesis

In the forestomach of ruminants, the cell wall and soluble carbohydrates of feedstuffs are degraded and fermented into volatile fatty acids. These fatty acids are shorter than 6 carbons long and are the main energy source for ruminants. The intake of diets containing more than 5 % of fat will inhibit forestomach fermentation and are not recommended for ruminants. Consequently, exogenous fatty acids are less evident in ruminant tissues when compared to non-ruminants (Boggs *et al.*, 1997). The main sites of fatty acid use are the mammary gland, subcutaneous adipose tissue and intramuscular lipid deposits of skeletal muscle adipocytes located in the perimysium and epimysium. The principal precursor for *de novo* fatty acid synthesis is acetate and not glucose (Bergen & Mersmann, 2005); irrespective of the carbon source, almost all fatty acids are produced - except for a few resulting from lower gut digestion of microorganisms - *via* endogenous synthesis in adipose and mammary glands during lactation only. The molecular regulation of *de novo* lipogenesis, lipid deposition and oxidation is affected by the same transcription factors and molecular mechanisms in both mammary tissue and subcutaneous fat stores (Bauman, Perfield, Harvatine & Baumgard, 2008; Harvatine, Perfield & Bauman, 2009). For example, during periods of rapid fat mobilization as often typical during the first month of lactation, hepatic accumulation of non-esterified fatty acids (NEFA) results first in ketosis followed by TAG synthesis (Pullen, Liesman, & Emery, 1990), but the TAG is not immediately derived from lipoproteins, and TAG accumulates in the liver resulting in a adipose tissue-lipid mobilization/NEFA release-dependent fatty liver. This problem is accentuated as bovine liver has a limited capacity for NEFA oxidation. While rates of liver TAG synthesis in ruminants are similar to those of non-ruminants, hepatic very low density lipoproteins (VLDL) secretion is very slow compared to non-ruminants (Bremmer, Bertics, & Grummer, 1999). Indeed, mechanistically hepatic steatosis in early postpartum dairy cows may be related to a lesser hepatic apoB availability

(Boggs *et al.*, 1987; Bernabucci *et al.*, 2004). Ruminant species not associated with high milk production are much less likely to develop fatty livers (Pullen *et al.*, 1990).

With beef cattle the emphasis in lipid metabolism has been on the extent of fat deposition and intramuscular lipid synthesis. Presently work is proceeding on the role of transcription factors on adipogenesis and fat synthesis in preadipocytes in muscle tissues, with both tissue culture and *in vivo* studies (Lengi & Corl, 2010). It appears that molecular regulation of preadipocyte differentiation in bovine muscle may be somewhat similar to the mechanisms described for 3T3-L1 adipocytes (Kokta *et al.*, 2004) in culture with key roles by CCAAT enhancer binding protein (C/EBP α,β,δ) and peroxisome proliferator-activated receptor (PPAR) (Hausman *et al.*, 2009; Yamada, Kawakami & Nakanishi, 2009; Mandrup & Lane, 1997). Thus, comparative differences of beef cattle present a unique resource to study various aspects of lipid metabolism.

1.5.2.1 Dietary fatty acids

The main fatty acid substrate for biohydrogenation in grazing animals is linoleic acid, as it is the most abundant fatty acid present in glycolipids and phospholipids of forages. Linoleic acid metabolism in the rumen involves the transient formation of CLA, mainly 18:2*c*9,*t*11, which is subsequently converted into vaccenic acid and finally stearic acid (Lourenço, Ramos-Morales & Wallace, 2010). The 18:2*c*9,*t*11 is usually the main CLA isomer found in beef and milk (Fritsche & Fritsche, 1998). Nonetheless, the 18:2*t*10,*c*12 becomes the predominant intermediate, particularly in high starch or oil-supplemented diets (Bauman & Griinari, 2001; Shingfield & Griinari, 2007).

1.5.2.2 *De novo* fatty acid synthesis

Fatty acid profiles are of utmost importance, not only for their physiological role, but also for their impact on the nutritional value of meat and the influence of long chain fatty acids on the consumer health. There are many factors that can effect fat deposition in the muscle of ruminants including anatomical location, breed, age, gender and diet. Ruminant diets normally have a moderate effect on the composition of adipose tissue because of ruminal hydrogenation.

There are at least three fatty desaturases in ruminant tissues, Δ^5, Δ^6, and Δ^9 desaturase. Of these, only the Δ^9 desaturase acts upon SFA to convert them to their respective MUFA. The Δ^9 desaturase, which is encoded by the SCD gene, also converts vaccenic acid to its corresponding CLA isomer, *c*9,*t*11 CLA (Smith *et al*., 2009a). Approximately 5% of the total

fatty acids in beef are comprised of polyunsaturated fatty acids (PUFA), by far the most abundant of which is linoleic acid (Smith *et al*., 2009a).

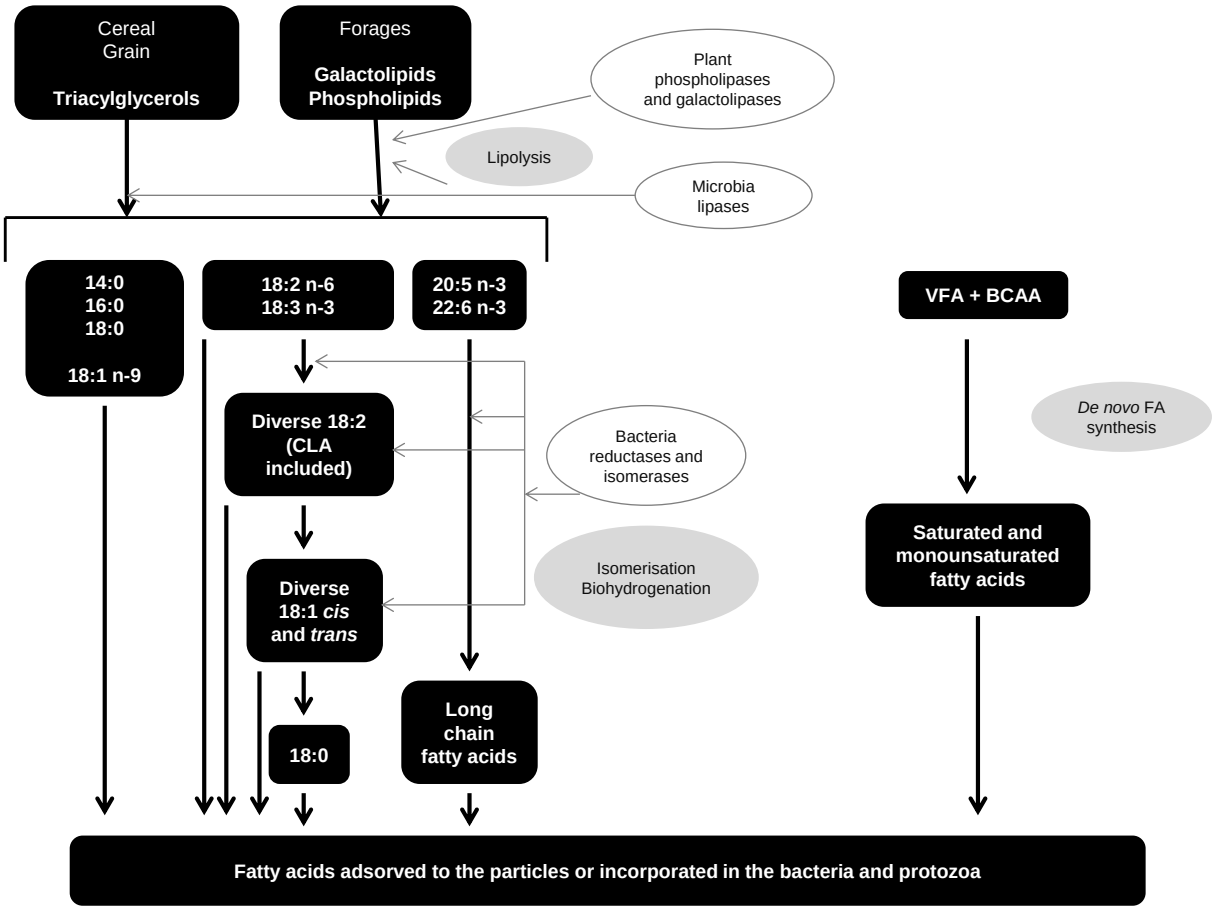

Figure 1.3 – Main steps of fatty acid metabolism in the rumen (BCAA, branched chain amino acids; CLA, conjugated linoleic acid; VFA, volatile fatty acids). Adapted from Harfoot (1981) and Collomb, Schmid, Sieber, Wechsler & Ryhänen (2006).

1.5.2.3 Rumen metabolism of fatty acids

The rumen hosts a complex ecosystem with an extremely high microbial density. Individual genera or species seldom have a unique role and overlapping of nutrient requirements and fermentation end-products often occurs. In addition, numerous interrelationships occur among the various ruminal microbes, such as symbiosis, competition, antagonism and predation, resulting in a highly competitive microbial community (Firkins, Karnati & Yu, 2008). The rumen microbial population digests the feed ingested by the ruminant host, and the resulting products of microbial digestion are the majority of nutrients that sustain the animal itself. Modifying the microbial fatty acid metabolism and profile by diet manipulation is a mechanism to improve the quality of ruminant-derived products, such as milk and meat.

However, the ruminal transformation of dietary lipid plays a major role in determining the fatty acid composition of ruminant products. In general, there are two main processes through which dietary lipids may be transformed in the rumen: lipolysis and biohydrogenation.

Rumen bacteria synthesize lipases that readily hydrolyse 18-carbon unsaturated a fatty acid found in lipids from forages and oilseeds. The extent of lipolysis in the rumen may be affected by diet composition (Faruque *et al.*, 1974 a,b; Singh & Hawke, 1979; Gerson, John, & King, 1986). The contribution of plant lipases to the overall process of lipolysis might be higher when actively growing pasture is fed. More recently, it was found that the primary factor affecting the rate of lipolysis of 18:2*n*-6 in soybean oil was the concentration of soybean oil in the culture medium (Beam *et al.*, 2000). Data indicate that lower fibre availability leads to reduced numbers of *Butirivibrio fibrisolvens*, which seem to be the primary lipolytic bacterium in the rumen under “normal” feeding conditions.

In recent years, interest has increased in branched-chain fatty acids (BCFA) due to demonstrated cytotoxic effects these biomolecules have on human cancer cells (Wongtangtintharn, Oku, Iwasaki, & Toda, 2004). The variations in the profile of odd- and branched-chain fatty acids (OBCFA) leaving the rumen are mainly a reflection of changes in relative abundance of specific bacterial populations (Vlaeminck, Fievez, Cabrita, Fonseca & Dewhurst, 2006; Fievez *et al.*, 2012). Several studies (Smith, Calder, Lough & Duncan, 1979; Manner, Maxwell & Williams, 1984; Humada, Serrano, Sañudo, Rolland & Dugan, 2012) have shown that BCFA arise from the digestion of enzyme consortia produced by ruminal bacteria, and that these fatty acids predominate in forage-fed cattle. Vlaeminck *et al.* (2006) described that these fatty acids can either originate from *de novo* synthesis in adipose tissue or from ruminal bacteria activity. Typically, concentrate-based diets are rich in starch and poor in neutral detergent fibre (NDF), leading to a decrease in rumen pH and fostering the growth of amylolytic bacteria while limiting the growth of cellulolytic bacteria (Weimer, Waghorn, Odt & Mertens, 1999; Vlaeminck *et al.*, 2006; Fievez, Colman, Castro-Montoya, Stefanov & Vlaeminck, 2012). The most important OBCFA from amylolytic bacteria are 15:0 and *a*15:0 (Fievez *et al.*, 2012). According to Vlaeminck *et al.*, (2006) rumen cellulolytic bacteria contain relatively high amounts of *iso*-fatty acids. It has been suggested that 15:0 and 17:0 fatty acids may reflect an increase in rumen propionate, regardless of their origin (bacterial or endogenous) (Fievez *et al.*, 2012). Fievez *et al.* (2012) showed that *i*15:0 was positively correlated to acetate. In addition, negative correlations were described for *i*15:0 and *i*16:0 and dietary starch contents (Vlaeminck *et al.*, 2006).

Lipolysis

The most important microbial transformations in the rumen are biohydrogenation and lipolysis. Esterified dietary lipids are rapidly and extensively hydrolysed by microbial lipases after feed ingestion, resulting in the release of constituent fatty acids (Jenkins, 1993) (Figure 1.3).

Recently, interest has revived in this ruminal metabolism because of the implications of microbial lipolysis on subsequent biohydrogenation of PUFA and generation of CLA (Lourenço *et al.*, 2010). Lipolysis results in a release of NEFA from esters to allow biohydrogenation. Therefore, a missing lipolysis may result in small quantities of dietary PUFA reaching the duodenum (Buccioni, Decandia, Minieri, Molle & Cabiddu, 2012).

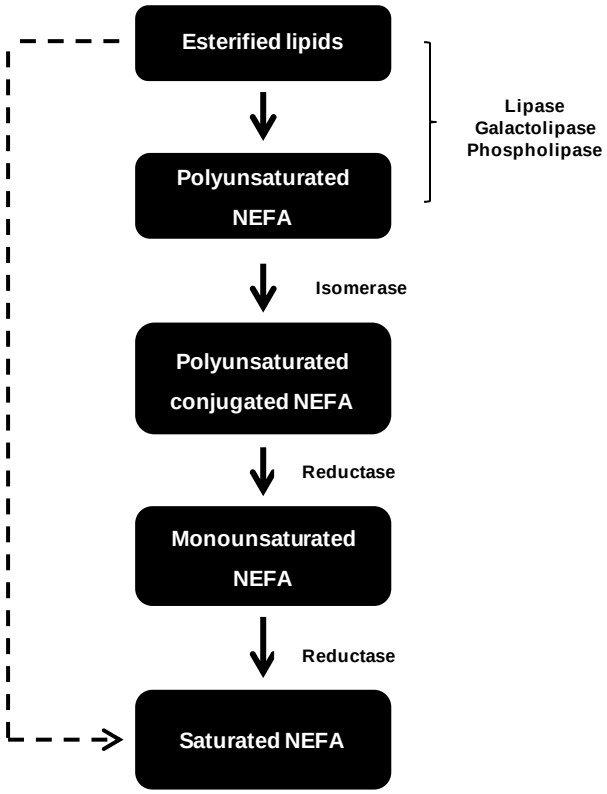

Figure 1.4 – Lipolysis and biohydrogenation. Adapted from Jenkins (1993).

Biohydrogenation

Reiser (1951) provided the first evidence of biohydrogenation in the rumen. Following incubation of 18:3*n*-3 with rumen fluid, increased accumulation of 18:2 isomers was observed. *In vitro* studies indicated that 18:0 accounted for 20% of the disappearance of 18:1*c*9, 18:2*n*-6, or 18:3*n*-3 when incubated with rumen contents from sheep (Shorland, Weenink, Johns & McDonald, 1957). *Trans*-18:1 isomers accounted for 17, 48, and 67% of

the original 18:1*c*9, 18:2*n*-6, or 18:3*n*-3 substrate. Linoleic acid, in particular, resulted in the formation of conjugated 18:2 isomers. *In vivo* studies later confirmed that 18:0 and 18:1 *trans* (45 and 34% of total rumen fatty acids) are the main products of hydrogenation (Wood, Bell, Grainger & Teckel, 1963).

Ruminal biohydrogenation may be simply described as a function of the available fatty acids pool size, ruminal retention time, and bacterial hydrogenation capacity (Harvatine & Allen, 2006). Bacterial hydrogenation is influenced by the species and concentration of the microbial population, as well as the ruminal environment (Harvatine & Allen, 2006).

- *Factors affecting biohydrogenation*.

The extent of biohydrogenation is determined by dietary fat source, retention time in the rumen, as well as the characterisitics of the microbial population (Allen, 2000). Allen Harvatine and Allen (2006) reported a linear decrease in 18:1 biohydrogenation, specifically *trans*-18:1, as the concentration of the unsaturated fat supplement increased. Hydrogenation of 18:1*c*9, 18:2*n*-6, and 18:3*n*-3 in diets with 50% silage (3.3 to 4.5% lipid) is extremely constant, and averages about 55, 76, and 81%, respectively (Doreau & Ferlay, 1994). However, the extent of hydrogenation of unsaturated fatty acids increases in proportion to their dietary intake. Supplementation with oils (2.5 to 10% of dry matter, DM) containing high concentrations of these fatty acids, increased the hydrogenation of 18:1*c*9 (38 to 73%), 18:2*n*-6 (70 to 95%), and 18:3*n*-3 (89 to 98%) (Murphy, Udén, Palmquist & Wiktorson, 1987; Doreau & Chilliard, 1997; Kalscheur, Teter, Piperova & Erdman, 1997; Wachira *et al.*, 2000). Hydrogenation of 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (DHA) averaged 75% in sheep fed a supplement of fish oil at 3% of DM (Wachira *et al.*, 2000).

- *Fatty acid intermediates during biohydrogenation*

Hydrogenation of 18:3*n*-3 and 18:2*n*-6 results in the formation of *trans*-monounsaturated fatty acids after the formation of *trans*-diene intermediates, which are rapidily metabolized (Harfoot & Hazlewood, 1997). *Trans*-18:1 may be hydrogenated to stearic acid (18:0) or passed to the duodenum (Harvatine & Allen, 2006). *Trans*-18:1 isomers produced during hydrogenation by *B. fibrisolvens* reflect the double bond positions of the substrates. Therefore, 18:1*t*11 is the main product of 18:2*n*-6 hydrogenation, despite accumulation of 18:1*t*9. Accumulation of 18:1*t*11 (46% of total fatty acids), 18:1*t*8 (27%), 18:1*t*10 (10%), 18:1*t*12 (9%) and 18:1*t*9 (7%) was observed when a mixture of 18:2*c*8,*t*10 (54%), *c*9,*t*11-18:2 (39%) and 18:2*t*10,*c*12 (3%) was incubates (Kepler, Irons, McNeill & Tove, 1966).

Dietary forage to concentrate ratio impacts on the ruminal outflow of *cis*-18:1, *trans*-18:1, and 18:0 of lactating cows. Feeding a low forage/high concentrate (25/75) diet without buffer, increased the flow of *cis*-18:1, *trans*-18:1, and 18:0 by 67, 97, and 22% compared with feeding a high forage/low concentrate (60/40) diet (Kalscheur *et al*., 1997). The low forage diet also lowered the hydrogenation of 18:2*n*-6 and 18:3*n*-3. Several studies indicate that high concentrate diets inhibit the growth of bacteria implicated in the reduction of *trans*-18:1 to 18:0 in the rumen (Tove & Matrone, 1962; Boeckaert *et al*., 2008). *In vitro* studies have shown the rapid biohydrogenation of 18:2 and 18:3, whereas a lower capacity for *trans*-18:1 biohydrogenation, particularly with decreased dietary fibre (Harfoot & Hazlewood, 1997). Taken together, these results are consistent with *in vitro* studies showing that hydrogenation of unsaturated fatty acids in the rumen leads to the accumulation of *trans*-18:1 isomers.

1.5.2.4 Adipose tissue metabolism of fatty acids

The amount of TAG stored within the adipocyte results from the equilibrium between *de novo* synthesis, uptake, esterification, lipolysis and reesterification of fatty acids (Chilliard, 1993). Desaturation of fatty acids involves the enzymatic removal of hydrogen from a methylene group in an acyl chain, an energy-requiring step that uses an activated oxygen intermediate. Free fatty acids must be esterified to coenzyme A prior to desaturation via stearoyl-CoA desaturase. Δ^9 desaturase is an iron-containing microsomal protein that catalyses the critical committed step in the biosynthesis of monounsaturated fatty acids by introducing the first *cis* double bond in the Δ^9 position of the carbon chain. The desaturase system involves three enzyme components: cytochrome b5, NADH-cytochrome b5 reductase, and a desaturase. Only the terminal desaturase activity is sensitive to changes in diet, hormonal balance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (Tocher, Leaver & Hodgson, 1998).

Theχ catalyses the desaturation of a large range of fatty acids. However, the preferred substrates of this enzyme are 16:0 and 18:0, which are converted to 16:1*c*9 and 18:1*c*9. The balance between 18:0 and 18:1*c*9 impact directly on membrane fluidity and the changes in this ratio have been implicated in several disease states, namely diabetes, obesity, hypertension, cancer, neurological, vascular, and heart disease (Tocher *et al.*, 1998). In bovine milk fat, a decrease in the ratios for 16:0 to 16:1*c*9 and 18:0 to 18:1*c*9 have also been used to interpret potential changes in desaturase activity due to dietary fatty acids (Griinari *et al.*, 2000).

Some trans18:1 isomers can be substrates for Δ^9 desaturase (Figure 1.5). Desaturation of 18:1*t*11 to 18:2*c*9,*t*11 was initially reported with liver homogenates from rats (Pollard,

Gunstone, James & Morris, 1980). Infusion of 18:1*t*11 into the abomasum of lactating cows also resulted in greater concentrations of 18:2*c*9,*t*11 in milk fat (Griinari *et al.*, 2000).

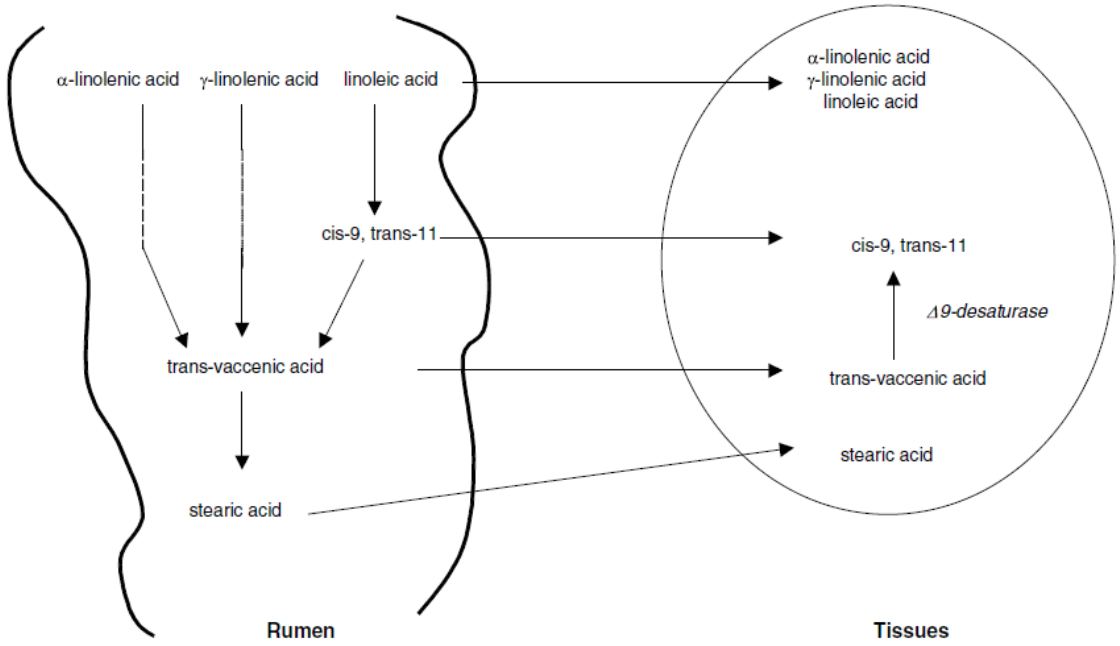

Figure 1.5 - Biosynthesis of *c*9,*t*11-18:2. Adapted from Schmid, Collomb, Sieber & Reed (2006).

It was estimated than >50% of milk fat 18:2*c*9,*t*11 was desaturated endogenously from 18:1*t*11 in the mammary gland. Given that 18:1*t*11 is the primary isomer arising during hydrogenation of 18:2*n*-6 and 18:3*n*-3, it has been hypothesised that its desaturation in the mammary gland is the primary source of milk fat 18:2*c*9,*t*11 (Griinari *et al.*, 2000). The increase in 18:2*c*9,*t*11 in mammary gland and milk fat due to feeding 18:1*t*11 to lactating mice, was associated with greater Δ^9 desaturase (Loor, Lin & Herbein, 1999) and mRNA abundance (Lin, Loor & Herbein, 2000). In beef cattle, 18:1*t*11 proportion was higher in the adipose tissue obtained from pasture-finished steers than feedlot-finished steers (Fincham *et al*., 2009). Daniel *et al*. (2004) proposed that SCD mRNA levels are increased with concentrate feeding in response to some component of the concentrate diet which is absent in the forage-based diets.

Expression of the Δ^9 desaturase gene and activity of the encoded enzyme are markedly reduced by 18:2*t*10,*c*12, but not 18:2*c*9,*t*11, in rodent adipose, liver, and mammary gland tissues (Choi, Enser, Wood & Scollan, 2000; Park *et al.*, 2000; Lin *et al.*, 2000). In contrast, 18:1*t*11 or 18:1*t*10 did not affect Δ^9 desaturase activity in the hepatic tissue (Park *et al.*, 2000). Initial studies evaluating the impact of CLA isomers in milk fat depression in dairy cows, indicated that 18:0 concentration in milk fat was substantially increased by abomasal infusion of CLA mixtures (Loor & Herbein, 1998; Chouinard, Corneau, Barbano, Metzger & Bauman, 1999). Studies indicate that, in addition to reducing milk fat percentage, 18:2*t*10,*c*12 also leads to a substantial increase in 18:0 concentration (Baumgard, Corl,

Dwyer, Saebo & Bauman, 2000). The negative effect on desaturation by 18:2*t*10,*c*12 might also be mediated by reductions in the transcription of the SCD gene, which were proportional to the concentration of CLA (Choi *et al.*, 2013).

1.5.3 Lipolysis

Lipolysis, that is, the mobilization of fatty acids from adipose tissue TAG occurs during times of negative energy balance or response to stress (Lass, Zimmermann, Oberer & Zechner, 2011). Basal lipolysis rates have been shown to increase with increasing fatness (Sidhu, Emery, Parr & Merkel, 1973; Pothoven, Beitz & Thornton, 1975). The decline in lipolysis observed during growth and fat accumulation is a consequence of adipocyte enlargement, with fewer cells in a given mass of tissue, rather than a decline in lipolytic activity of the individual adipocytes (Pothoven *et al.*, 1975).

Mobilisation of adipose tissue lipids to be used elsewhere in the body requires the hydrolytic cleavage of the TAG to NEFA and glycerols. The cellular concentration of NEFA is a tightly regulated balance between the hydrolysis of TAG and the esterification of the NEFA (Lass *et al*., 2011). Consistent with its central importance in lipid and energy homeostasis, lipolysis occurs in essentially all tissues and cell types, although it is more effective in white and brown adipose tissues (Lass *et al.*, 2011). To date, three enzymes have been implicated in the complete hydrolysis of TAG molecules in cellular lipid stores: adipose triglyceride lípase, hormone-sensitive lipase and monoglyceride lipase (Zimmermann *et al.*, 2004; Lass *et al*., 2011).

Nutrient supply to the adipose tissue appears to control capacities of both the lipogenic and lipolytic capacity. In fact, in studies comparing fat deposition in bulls fed *ad libitum* with those fed restrictely a similar degree of fat deposition was observed (Pothoven, *et al.*, 1975; Henricks, Jenkins, Ward, Krishnan & Grimes, 1994), suggesting that lipolysis rates were decreased, in conjunction with the lipogenic rates, with feed restriction.

1.5.4 Oxidation and ketogenesis

The liver takes up NEFA from blood in proportion to their concentration. Within the hepatocytes, long chain fatty acids of 14 carbons or more are activated by acyl-CoA synthases found in the microsomes and outer mitochondrial membrane (Drackley, 2000). Under conditions of increased fatty acid uptake, the liver often produces large amounts of ketone bodies, acetoacetate and β-hydroxybutyrate, in a process designated by ketogenesis.

The two main factors regulating the degree to which fatty acids are oxidized by the liver are the supply of fatty acids to the liver via lipolysis and the partitioning within hepatocytes between mitochondrial oxidation and microsomal esterification.

The enzyme carnitine palmitoyltransferase I (CPT-I), located on the outer mitochondrial membrane, regulates the entry of the fatty acids with 14 carbons or more into the mitochondria. Short- and medium-chain fatty acids (12 carbons or less) pass through the mitochondrial membrane and are not controlled by CPT-I. Ketogenesis is promoted in times of increased fatty acid mobilization and uptake by the liver, when low rations of insulin to glucagon cause activation of CPT-I, which allows extensive uptake of fatty acids into the mitochondria (Zammit, 1990).

An alternate pathway for β-oxidation in liver is found in peroxisomes (Singh, 1997), functioning in a similar way to the mitochondrial pathway. Peroxisomal β-oxidation is active with very long chain fatty acids (20 carbons or more) that are relatively poor substrates for mitochondrial β-oxidation. Studies in ruminants (Grum, Hansen, & Drackley, 1994; Cherfaoui *et al*., 2012) indicated that the liver from these species possess relatively high peroxisomal β-oxidation activity.

1.5.5 Hepatic lipid metabolism

The liver takes up free fatty acids from the blood in proportion to their concentration. Within the hepatocytes, fatty acids longer than 14 carbons are activated by acyl-coA synthases found in the microsomes and outer mitochondrial membrane. The two main factors regulating the degree to which fatty acids are oxidized by the liver are the supply of fatty acids to the liver via lipolysis and the partitioning within hepatocytes between mitochondrial oxidation and microsomal esterification. Long chain fatty acids (LCFA) entering the liver are derived from plasma NEFA mobilized from adipose tissues during fasting conditions or from hydrolysis of dietary TAG during the post-prandial period. The hepatic uptake of NEFA is driven by mass action and therefore depends in their concentration and the rate of blood flow into the liver (Pethick, Bell & Anninson, 1984).

The metabolism of LCFA in the liver depends on the feeding conditions. Fatty acid metabolism in the liver is also under hormonal control, as insulin inhibits the oxidation of fatty acids but stimulates the formation of LCFA from glucose and the synthesis of TAG from LCFA (Zammit, 1995).

1.5.5.1 Desaturation of Long-Chain Fatty Acids

There is some controversy sorrounding the mechanism of DHA biosynthesis in mammals. Classically, it was accepted that DHA and 22:5*n*-6 are formed by Δ^4-desaturation of their immediate precursors 22:5*n*-3 and 22:4*n*-6, respectively, by introducing a double bond in position Δ^4 (Martinez *et al*., 2010) (Figure 1.6). However, this pathway was practically abandoned in mammals and nowadays an alternate route involving the peroxisome is generally accepted (Sprecher, 2000). This route implies the microsomal elongation of 22:5*n*-3 to 24:5*n*-3, and a second Δ^6-desaturation step to 24:6*n*-3, which would finally be β-oxidated in the peroxisome to yield DHA. The same route would work for the *n*-6 family, producing 22:5*n*-6 from 24:5*n*-6. The existence of a Δ^4-desaturase has been demonstrated in lower eukaryotes and, recently, in a vertebrate species (Li *et al*., 2010). Nevertheless, it remains unkown whether or not there is an intrinsic Δ^4-desaturase in ruminants.

Δ^6 desaturase has an important role in animals because it catalyses the rate limiting step in the desaturation and elongation of the essential fatty acids, 18:2*n*-6 and 18:3*n*-3, to 20:4*n*-6 and 22:6*n*-3, respectively (Tocher, Leaver & Hodgson, 1998). Δ^5 desaturase activity is coupled with that of Δ^6 desaturase, because it catalyses the desaturation of 20:4*n*-3 and 20:3*n*-6 to 20:5*n*-3 and 20:4*n*-6.

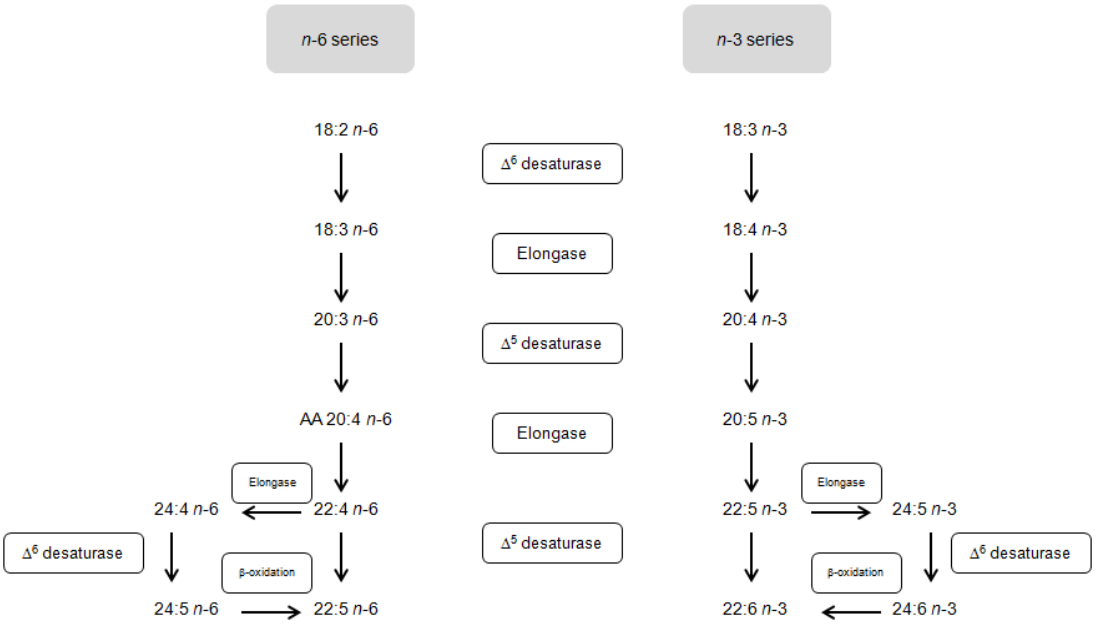

Figure 1.6 – Metabolic pathways of the *n*-6 and *n*-3 series. Adapted from Li *et al*. (2010)

Δ^5 desaturase and Δ^6 desaturase are iron-containing microsomal proteins. The desaturase system involves three enzyme components: cytochrome b5, NADH-cytochrome b5 reductase, and a desaturase. The two electrons needed are transported through an electron-transport system that is composed of cytochrome b5 and NADH-dependent cytochrome b5

reductase. Activity of the desaturase is sensitive to changes in dietary protein and 18:2*n*-6 availability. Despite similarities with Δ^9 desaturase, they are different enzymes (Tocher *et al.*, 1998).

Data regarding the activity of Δ^6 or Δ^5 desaturase in ruminant tissues is relatively scarce. Nevertheles, studies using abomasal infusions to by-pass hydrogenation, it was shown that the concentrations of 20:4*n*-6, 20:5*n*-3, and 22:6*n*-3 in milk fat increased proportionaly to the amount of 18:2*n*-6 or 18:3*n*-3 available for uptake (Hagemeister, Precht, Franzen & Barth, 1991; Loor & Herbein, 1998).

The potential of CLA to decrease the activity of Δ^6 and/or Δ^5 desaturase in the mammary gland of cows has been demonstrated. The concentration of 20:4*n*-6 in milk fat decreased inversely with CLA (18:2*c*9,*t*11 and 18:2*t*10,*c*12 isomers) in spite of exogenous 18:2*n*-6 being available (Loor & Herbein, 1998). A similar response was observed in subcutaneous and omental adipose tissue of piglets suckling from sows consuming a CLA mixture, compared with 18:2*n*-6 (Bee, 2000). In the rat hepatic tissue, 18:2*c*9,*t*11, compared with 18:2*t*10,*c*12 reduced the desaturation of 18:2*n*-6 or 18:3*n*-3 through a decrease in the activity of Δ^6 desaturase (Bretillon, Chardigny, Grégoire, Berdeaux, & Sébédio, 1999). However, a concentration four times higher than 18:2*c*9,*t*11 was necessary for 18:2*t*10,*c*12 to inhibit the desaturation of 18:2*n*-6.

1.6 Endocrine regulation of lipid metabolism

Until the 1980s, fat cells were viewed almost exclusively as a depot for the storage of energy, with lipids being added or released under the influence of the appropriate hormones (Rosen & Spiegelman, 2000). This mechanism was reported to be regulated by hormones and various adipogenic transcription factors which are expressed as a transcriptional cascade promoting adipocyte differentiation and leading to the mature adipocyte phenotype (Romão *et al.*, 2011).

It is now recognised that more than storing the body's fuel reserves, providing insulation and padding, the adipose tissue is a dynamic endocrine organ (Galic, Oakhill & Steinberg, 2010; Poulos, Hausman & Hausman, 2010). As such, the secretions from adipose tissue are known to affect several systems such as the vascular and immune systems and play major roles in metabolism (Poulos *et al.,* 2010). The adipose tissue is now accepted to be an endocrine organ that secretes numerous hormones, growth factors, matrix proteins, enzymes, cytokines, and complement factors (Figure 1.7).

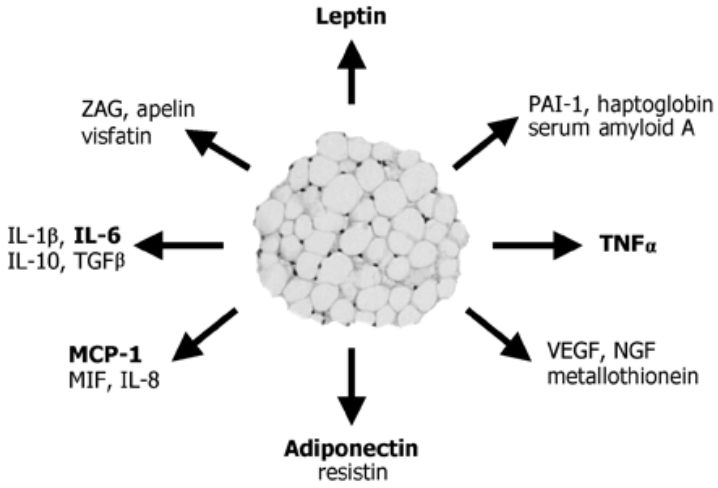

Figure 1.7 - Major adipokines secreted from white adipose tissue. *A*dapted from Trayhurn *et al.* (2006).

1.6.1 Insulin

The role of insulin in ruminant adipose tissue metabolism remains to be clearly established. While some authors provided evidence to support its importance (Rhoades *et al.*, 2007; Rhoades, Sawyer, Ponce, Lunt & Smith, 2009), data from *in vitro* studies suggest that fat acid synthesis in bovine subcutaneous adipose tissue is not sensitive to insulin levels (Vernon *et al.*, 1985; Miller *et al.*, 1991).

Insulin stimulates glucose synthesis as well as the entrance of amino acids in the cells. The activity of lipoprotein lipase in the adipose tissue, and subsequent increase in the deposition of TAG, is promoted by insulin (Wang & Eckel, 2009). However, the lipoprotein lipase (LPL) activity in the muscle tissue does not appear to be insulin-dependent (Wang & Eckel, 2009).

1.6.2 Leptin

The identification and characterization of the biological role of leptin led to the recognition that white fat is an important endocrine organ (Trayhurn, Bing & Wood, 2006). The white adipose tissue is the main site for leptin synthesis (Margetic, Gazzola, Pegg & Hill, 2002). Plasma levels of leptin have been associated with the extension of the adipose tissue in several species (Benoit, Clegg, Seely & Woods, 2004), including beef cattle.

1.6.3 Adipokines

The adipokines encompass classical cytokines (*e.g.*, TNF-*α*; interleukin-6, IL-6), chemokines (*e.g.*, monocyte chemoattractant protein-1, MCP-1), proteins of the alternative complement system (*e.g.*, adipsin), as well as proteins involved in vascular haemostasis (*e.g.*, plasminogen activator inhibitor-1, PAI-1), the regulation of blood pressure (angiotensinogen), lipid metabolism (*e.g.*, cholesteryl ester transfer protein, retinol binding protein), glucose homeostasis (*e.g.*, adiponectin), and angiogenesis (*e.g.*, vascular endothelial growth factor, VEGF) (Trayhurn *et al.*, 2006). Adipokines are implicated in a wide range of pathophysiological processes, namely related controlling energy balance and lipid metabolism (Kershaw & Flier, 2004). Excess adipose tissue is associated with elevated plasma levels of adipokines, except for adiponectin.

1.7 Gene regulation of lipid metabolism

Adipogenesis in mammals is regulated both hormonally and genetically. Adipogenic transcription factors, which regulate the expression of many adipogenic genes leading to the differentiation of adipocytes have been identified (Figure 1.8), such as peroxisome proliferator-activated receptor-element-binding protein (SREBP). Adipogenesis is a process regulated by a complex cascade of transcription factors (Ayala-Sumuano *et al.*, 2011). The peroxisome proliferator-activated receptor gamma (PPARγ) is an adipogenic protein which was shown to regulate pre-adipocyte differentiation in rodents (Desvergne, Michalik & Wahli, 2006).

The sequential expression of certain specific transcription factors, including the CCAAT/enhancer-binding protein (CEBP), the SREBP and those of the peroxisome proliferator-activated receptor (PPAR) families, plays a key role in the early stages of adipocyte conversion (Gregoire, Smas & Sul, 1998).

In vitro induction of bovine perimuscular pre-adipocytes has indicated that the expression of *PPARG*, *SREBF1*, fatty acid binding protein 4 (*FABP4*) and fatty acid synthase (*FASN*) gene expression is increased (Taniguchi *et al.*, 2008). Moreover, Taniguchi *et al.* (2008) suggested that *PPARG* and *FASN* are essential to sustain the differentiation programme. The strict requirement of SREBF1 for adipogenesis and lipogenesis in cattle is unknown. A recent study in rodents found that lipogenic gene expression in adipose tissue was independent of SREBF1 (Sekiya *et al.*, 2007).

During differentiation, subcutaneous and visceral preadipocytes showed different, and depot-specific, effects on the expression of C/EBP, carnitine palmitoyl transferase 1B (CPT1B) and FABP4 (Kokta *et al.*, 2004). Using sheep as a model, it was determined that visceral-derived preadipocytes were less able to differentiate than subcutaneous-derived preadipocytes (Soret, Lee, Finley, Lee& Vernon, 1999). A recent study performed by Yamada *et al.* (2009) in cattle demonstrated differences in the expression of the adipogenic transcription factors of the C/EPB family in subcutaneous and intramuscular adipose tissue, depending on the dietary roughage level.

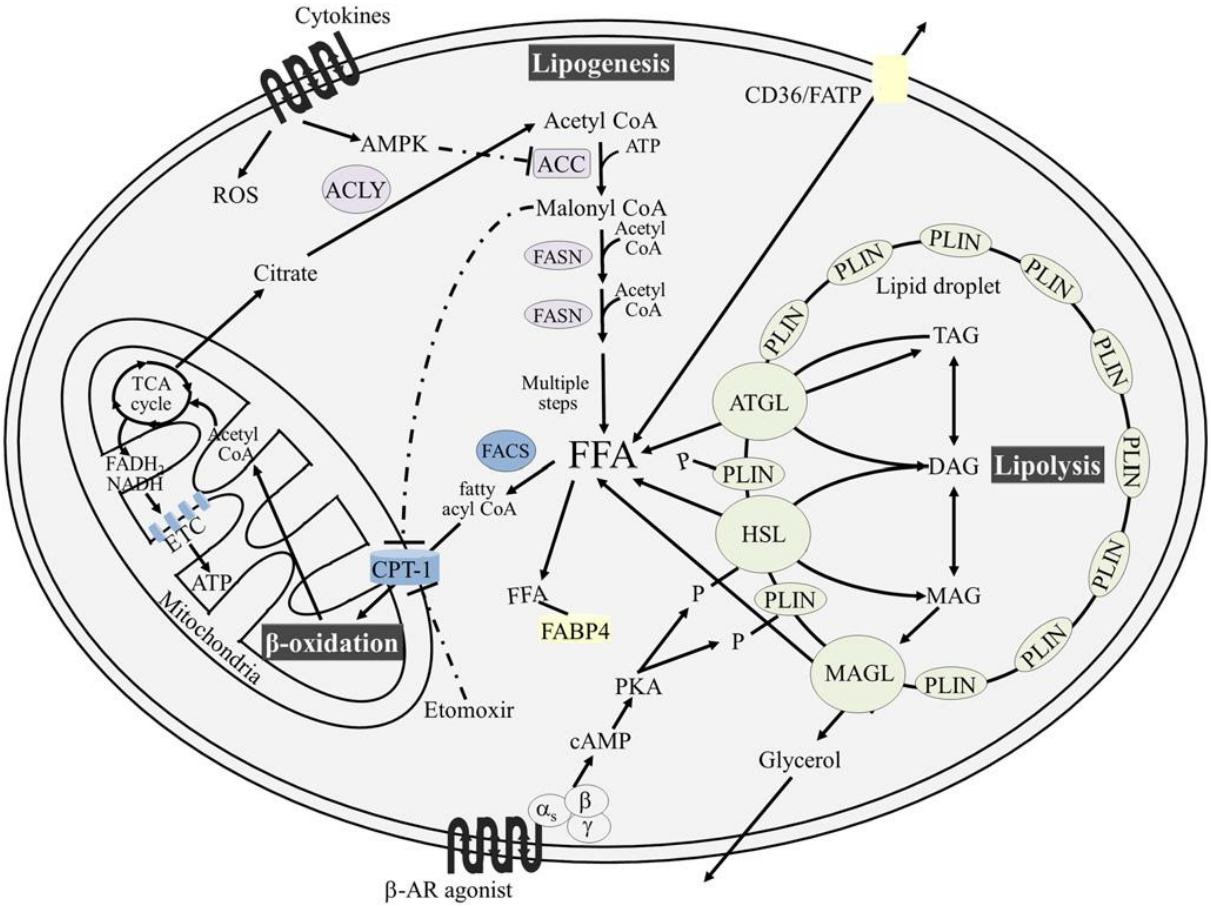

Figure 1.8 – Overview of adipocyte lipid metabolism. *A*dapted from Nieman *et al.* (2013).

Adipocyte differentiation requires a plethora of transcription factors. The PPARγ and members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (Morrison & Farmer, 2000; Rosen, Walkey, Puigserver & Spiegelman, 2000) are considered key during the differentiation process. When the adipogenic stimulation is complete, the expressions of *CEBPD* and *CEBPB* are induced in early phase, and the ones of *CEBPA* and

PPARG2 are expressed later by transcriptional regulation (Tanaka, Yoshida, Kishimoto & Akira, 1997; Rosen *et al*., 2000). These two transcription factors, C/EBPα and PPARγ2, are believed to play a central role for the control of adipocyte-specific gene expression. (Tang, Otto & Lane, 2003).

The reaction catalysed by acetyl-CoA carboxylase alpha (ACACA) takes place in two steps: (1) carboxylation of biotin and (2) transfer of the carboxyl to acetyl-CoA to form malonyl-CoA. ACACA is a multienzyme protein, containing a variable number of identical subunits, each containing biotin, biotin carboxylase, biotin carboxyl carrier protein, and transcarboxylase, as well as a regulatory allosteric site.

Mammalian FASN is a multi-enzyme complex that may not be subdivided without loss of activity, and the acyl carrier protein is part of this complex. The aggregation of all the enzymes of FASN into one multienzyme functional unit offers great efficiency and freedom from interference by competing reactions. Another advantage of a single multienzyme complex is that synthesis of all enzymes in the complex is coordinated, since a single gene encodes it.

Trans-18:1 isomers, however, target ACACA and FASN directly. Greater flow of total *trans*-18:1 isomers to the duodenum was negatively correlated with milk fat percentage and de novo fatty acid synthesis (Wonsil, Herbain & Watkins, 1994; Kalscheur *et al.*, 1997). However, an increase in the proportion of the 18:1*t*10, not 18:1*t*11, isomer in milk fat was correlated with the decreases observed (Griinari *et al.*, 1998; Piperova *et al.*, 2000). Lower activity and mRNA abundance for ACACA and FASN were responsible for the reduction in lipid synthesis in the mammary gland due to greater concentrations of 18:1*t*10 (Piperova *et al.*, 2000).

In beef, most of the MUFA are created by the conversion of SFA in the adipose tissue of the animal (Smith, Gill, Lunt & Brooks, 2009a). This function is regulated by the *SCD* gene (Chung, Choi, Kawachi, Yano & Smith, 2006). There is evidence that *SCD* gene expression may also be affected by age, as older animals usually have higher MUFA:SFA ratios (Chung *et al.*, 2006). A product with a higher MUFA:SFA ratio would be able to provide domestic consumers with a fatty acid profile that is more in-line with the changes in the American Heart Association guidelines (Krauss *et al.*, 2001). Increase in MUFA is correlated with increased fat softness and increased muscle marbling (Wood *et al.*, 2008).

PPARγ, a transcription factor that acts as an important regulator of lipid metabolism and energy homeostasis, plays a key role in the control of the pathways involved in fatty acid uptake, fatty acid binding, fatty acid oxidation, ketogenesis, as well as carnitine synthesis (Desvergne & Wahli, 1999). Particularly well studied in rodents, PPARα and PPARγ serve as master regulators of hepatic fatty acid oxidation and adipose tissue insulin sensitivity (Mandard, Müller & Kersten, 2004; Desvergne *et al.*, 2006; Loor, 2010). PPARγ plays an

important role in regulating adipose metabolism of glucose and long chain fatty acids (LCFA) and reduces inflammation (Hauner, 2002; Stienstra *et al.*, 2007). A central role in endogenous oleic acid synthesis via SCD for adipose tissue TAG synthesis could be advanced. However, there is no statistical correlation between expression patterns of genes involved in desaturation and desaturase indexes (Δ^5, Δ^6, Δ^9), rendering their use to infer temporal enzyme expression/activity meaningless (Loor, 2010).

Fatty acid-binding proteins can bind and transport saturated and unsaturated fatty acids to adipocytes for lipid synthesis. Therefore, increased expression of these genes may promote fat deposition (Jin *et al.*, 2012). The fatty acid-binding protein 4 (FABP4), a lipid chaperone, is expressed in both adipocytes and macrophages. The *FABP4* and *SCD* genes are specifically expressed in adipose tissue (Hu, Liang & Spiegelman, 1996; Wu *et al.*, 2009), so it is possible that the increased expression derived simply from an increased amount of adipose deposition.

Fatty acid availability influences the extent of IMF deposition (Bong *et al.*, 2012). The regulation of fatty acid influx into the myocyte to provide the necessary substrate for IMF synthesis is, therefore, important. LPL is a rate limiting enzyme for the hydrolysis of the TAG cores of circulating TAG-rich lipoproteins. LPL-catalysed reaction products, fatty acids and monoacylglycerol, are taken up, in part, by adipose tissue and skeletal muscle and stored as neutral lipids (Wang *et al.*, 2009). TAG are transported in the form of VLDL. The NEFA and lipoproteins remnants are among the products of LPL-mediated lipolysis (Tschernatsch, Mlecnik, Trajanoski, Zechner & Zimmermann, 2006). LPL activity, being related to the release of lipolytic products from chylomicra and VLDL to the adipocytes for deposit as TAG, is a key regulator of fat accumulation in various adipose tissue depots (Wajchenberg, 2000).

Carnitine acetyltransferases catalyse the exchange of acyl groups between carnitine and coenzyme A (CoA) (Jogl, Hsiao & Tong, 2004). These enzymes include carnitine acetyltransferase (CRAT) and the carnitine palmitoyltransferases (CPT). CPT-I and CPT-II are crucial for the β-oxidation of long-chain fatty acids into the mitochondria by enabling their transport across the mitochondrial membrane. CPT-I executes the initial step of the oxidation of long chain acyl-CoAs by catalysing the reversible transesterification of long chain acyl-CoA with carnitine (Noland *et al.*, 2009). CRAT is a mitochondrial enzyme that converts short chain CoA species into their acylcarnitine counterparts while also regenerating free CoA (Cordente *et al.*, 2004). Accordingly, CRAT is presumed to play a key role in regulating the activities of mitochondrial enzymes that respond to the acetyl-CoA/CoA ratio, such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Ramsay & Zammit, 2004). It has been speculated that chronic lipid stress might lead to a specific compromise in the intramitochondrial pool of free carnitine, which in turn antagonizes CRAT activity, mitochondrial function, glucose/pyruvate oxidation and insulin action (Noland *et al.*, 2009).

To date, six enzymes, termed ELOVL1-6 (elongation of very long chain fatty acid) have been identified and suggested to perform the condensation reaction in the elongation cycle. ELOVL2 was hypothesized to have a role in PUFA synthesis on the basis of complementation studies in yeast (Tvrdik *et al.*, 2000). Subsequently, both mouse and human ELOVL2 were shown to elongate arachidonic acid (20:4*n*-6), eicosapentaenoic acid (20:5*n*-3), docosatetraenoic acid (22:4*n*-6) and docosapentaenoic acid (22:5*n*-3) in transfected yeast and mammalian HEK293 cells, whereas no activity was detected for saturated/monounsaturated substrates (Moon, Shah, Mohapatra, Warrington, Horton, 2001; Leonard *et al.*, 2002). ELOVL5, is involved in the elongation of various polyunsaturated long-chain fatty acids of C18–C20 (Leonard *et al.*, 2000). However, ELOVL5 does not appear to have the capacity to elongate PUFA substrates beyond C22. Although ELOVL5 expression can be detected in most human tissues, the highest mRNA levels were found in the testis and adrenal gland, consistent with the fact that these two tissues contain high levels of docosapentanoic acid (22:5*n*-6), a PUFA metabolite (reviewed by Jakobsson, Westerberg & Jacobsson, 2006).

In humans, studies showed that DNA polymorphisms in fatty acid desaturase 1 gene (FADS1) family were associated with arachidonic (20:4*n*-6), linoleic (18:2*n*-6), alpha-linolenic (18:3*n*-3) and eicosadienoic (20:2*n*-6) acids of human serum (Malerba *et al.*, 2008). The fatty acid desaturase 1 gene (FADS1) has been considered as one of the rate-limiting enzymes to the endogenous formation of LC-PUFA in humans (Lattka, Illig, Koletzko, & Heinrich, 2010). In mammals FADS1 converts dihomo-γ-linolenic acid (20:3*n*-6) to arachidonic acid (20:4*n*-6) and eicosatetraenoic acid (20:4*n*-3) to eicosapentaenoic acid (20:5*n*-3) with linoleic (18:2*n*-6) and linolenic (18:3*n*-3) acids as the initial substrate (Lattka *et al.*, 2010; Schaeffer *et al.*, 2006).

The liver is the main gluconeogenic organ, and 90% of the glucose in ruminants is provided by hepatic gluconeogenesis. The main role of insulin in the liver, muscle and fat, by acting through the cell surface insulin receptor (INSR), is to stimulate glucose uptake into hepatocytes to promote glycogen synthesis and to maintain blood sugar homeostasis (Biddinger, 2006; Vikram & Jena, 2010). The regulation of energy metabolism, including gluconeogenesis, fat mobilization and glycogen storage/breakdown in ruminants is mainly regulated by insulin and glucagon (Balogh *et al.*, 2008). Accordingly, the regulation of the InsR has become a focus of ruminant feeding and energy metabolism research.

1.8 Research objectives

Fat deposition has been extensively studied in beef cattle in the last decades, with particular emphasis on the effect of breed and diet composition on the development of the main carcass fat depots. Nonetheless, the mechanisms underlying this process are far from well understood. The main objective of this study was, therefore, to elucidate the effect of genetic background and dietary silage level on fat deposition and partitioning, as well as to investigate the genetic, metabolic and endocrine mechanisms involved.

The research was carried out in two Portuguese autochthonous bovine breeds (Alentejana and Barrosã) fed on distinct dietary silage levels (30% *vs.* 70%) from 8 to 18 months old. The detailed objectives of the present work were as follows:

a) to study the effect of high and low silage diets on the productive traits and carcass composition of Alentejana and Barrosã young bulls;
b) to evaluate the effect of breed and diet on the cellularity and fatty acid profile of subcutaneous and mesenteric fat depots;
c) to assess the influence of breed and diet on the levels of gene expression of key adipogenic/lipogenic factors of muscle and subcutaneous adipose tissue, and its implications on tissues fatty acid composition;
d) to study the influence of the genetic background and dietary silage level on meat fatty acid composition, as well as on neutral and polar lipids fractions;
e) to address the hepatic regulation of lipid metabolism, with particular emphasis on the biosynthesis of long chain *n*-3 PUFA.

CHAPTER 2

Productive traits and carcass composition

Carcass fat partitioning and meat quality of Alentejana and Barrosã young bulls fed high or low maize silage diets

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ASH Costa participated in the slaughter sampling, did the laboratory analysis, data processing and statistical analysis. In addition, ASH Costa collaborated in the interpretation of the results and writing of the manuscript.

Abstract

This study assessed the effect of breed and diet on carcass composition, particularly fat partitioning, and meat quality in young bulls. An experiment with forty young bulls from two phylogenetically distant Portuguese bovine breeds, Alentejana and Barrosã, fed two diets with different maize silage to concentrate ratios, but isoenergetic and isonitrogenous, was carried out until the animals reached 18 months of age. In the LL muscle, Barrosã bulls fed the low silage diet had the highest IMF content. Bulls fed the low silage diet also had the highest IMF content in the *semitendinosus* muscle. Diet determined the proportions of total visceral fat and individual fat depots. Under these experimental conditions, it was shown that the genetic background is a major determinant of carcass composition and meat quality, and that the dietary differences studied had limited effect on carcass composition.

2.1. Background

Carcass composition reflects the differential growth pattern of the major component tissues and determines the commercial value of carcasses in meat-producing animals (Berg, Andersen, & Liboriussen, 1978). In fact, the weight, proportion and carcass distribution of fat, bone and muscle have a high economic impact on beef cattle production (Kempster, 1986). In addition, IMF has become a key factor of carcass quality for the beef industry, which, in order to comply with the European market requirements, has shifted towards the production of lean beef.

A large amount of subcutaneous and visceral adipose tissues, regarded as "waste fat", is deposited in parallel to IMF, or "taste fat", during the maturing phase of cattle (Fiems *et al.*, 2000; Gotoh *et al.*, 2009). The current beef production is focused on reducing the deposition of "waste fat", while maintaining high meat sensory and nutritional quality standards. Genetic and environmental factors, particularly nutrition, determine fat partitioning among the subcutaneous, intermuscular and visceral depots (Kempster, 1986). Therefore, it is important to quantify each fat depot proportion in the carcass. This is particularly relevant for IMF due to its contribution to eating quality, which should not be compromised (Savell & Cross, 1988). Thus, the development of strategies to manipulate adipose tissue deposition in farm animals has been one of the major breeding goals for some years (De Smet, Raes, & Demeyer, 2004). Nonetheless, the relationship between the deposition of IMF and "waste fats", as well as the patterns of their changes during growth, remain rather unclear (Aldai, Nájera, Dugan, Celaya, & Osoro, 2007; Gotoh *et al.*, 2009).

A wide range of factors influence meat quality of ruminant animals. Insights on the relationship among productive traits, carcass composition and meat quality would contribute to improve beef production through genetic selection, as well as to understand the final product acceptability (Piedrafita *et al.*, 2003). Much has been hypothesized about the role of diet on animal performance and meat quality, although the results are often contradictory. While some authors reported negative effects of silage-based diets on productive and carcass traits compared to concentrate-based diets (Priolo, Micol, & Agabriel, 2001), others found slight (Blanco *et al.*, 2010) or no differences at all (French *et al.*, 2001) between feeding strategies. Moreover, breed has been referred to as one of the main factors influencing feed intake, growth rate and, consequently, carcass composition (Albertí *et al.*, 1998; Clarke *et al.*, 2009) and beef quality (Chambaz, Scheeder, Kreuzer & Dufey, 2003; Bartoň, Bureš, and Kudrna 2010; Bressan *et al.*, 2011). However, the effect of genotype on meat quality has yielded conflicting results because some studies failed to demonstrate differences in meat between breeds (Muir, Wallace, Dobbie & Bown, 2000; Vieira, Cerdeño, Serrano, Lavín & Mantecón, 2007). These apparently contradictory results should be

interpreted with caution because some of the studies compared breeds selected for meat production and/or with similar genetic background, whereas others used breeds which differed in purpose (milk, meat or both), growth rate and mature weight.

Previous studies from our research group found differences in lipid content, composition and nutritional quality between beef obtained from Alentejana and Barrosã breeds produced with distinct management (Alfaia *et al.*, 2007, 2009; Costa *et al.*, 2010), with consistently higher IMF contents for the Barrosã breed. However, it must be taken into account that in these works the animals were reared according to their local production systems. On the other hand, published information regarding the productive and carcass traits of these two phylogenetically distant Portuguese bovine breeds (Beja-Pereira *et al.*, 2003), with different frame size and precociousness, remains scarce. Works by Silva, Lemos, Monteiro and Portugal (1998), and Simões and Mira (2002) compared Alentejana and Barrosã breeds reared under experimental intensive conditions. Silva *et al.* (1998) found that as Alentejana young bulls approach maturity the deposition of fat in internal depots increases, whereas in the Barrosã breed fat was preferentially deposited in subcutaneous and intermuscular depots. In contrast, Simões and Mira (2002) observed that these breeds tend to show similar fat partitioning, when compared at the same total carcass fat.

To our knowledge, there are no previous studies comparing the effect of different dietary maize silage/concentrate ratios on Alentejana and Barrosã carcass and meat traits, under controlled experimental conditions. We hypothesized that the comparison of a large-framed breed (Alentejana) with a small-framed breed (Barrosã), at the same age, would reveal different carcass composition and fat partitioning. In addition, the use of isoenergetic and isonitrogenous diets with different maize silage/concentrate ratios (30/70% *vs.* 70/30%) would add depth to the study of the carcass fat distribution, thus contributing to the knowledge on the features of adipose tissue deposition. Therefore, this study aimed to assess the effect of breed and diet on the carcass composition, particularly fat partitioning, and meat quality of the two Portuguese autochthonous breeds, Alentejana and Barrosã, fed high or low silage diets.

2.2. Material and Methods

The experimental trial was conducted at the facilities of Unidade de Produção Animal, L-INIA, INIAV (Fonte Boa, Vale de Santarém, Portugal), from January to November 2009. All animals were handled in accordance with local and national guidelines covering animal experiments, reviewed by the Ethics Commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária) following the appropriate European Union guidelines (Directive 86/609/EEC).

2.2.1 Animals, feeding and performance

Twenty purebred young bulls from Alentejana (large-framed) and twenty Barrosã (small-framed) breeds were assigned to either high or low silage diets (four experimental groups of 10 animals each, *n*=10). The animals were housed in eight adjacent pens, two pens per breed and diet. Diets were approximately isoenergetic and isonitrogenous, and composed of 30/70% (low silage, LS) and 70/30% (high silage, HS) of maize silage and concentrate, respectively, on a DM basis. The ingredients and chemical composition of diets are described in Table 2.1.

Samples of the diets were collected three times during the course of the experimental trial (*n*=3). Feed samples were analysed for DM by drying a sample at 100 ºC to a constant weight. Nitrogen content was determined by Kjeldahl (AOAC, 1990) and crude protein was calculated as 6.25×N. NDF and acid detergent fibre (ADF) were determined by the procedure of Van Soest, Robertson and Lewis (1991). The samples were extracted with petroleum ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany), to determine crude fat. Determination of ash and starch contents was carried out according to the procedures described by the AOAC (1990) and Clegg (1956), respectively. Gross energy in the feed was determined by adiabatic bomb calorimetry (Parr 1261, Parr Instrument Company, Moline, IL, USA).

Bulls were housed in eight pens, two pens *per* breed and diet. Replicate pens within a treatment were positioned in different parts of the facility. The initial age was 11 ± 1.0 months and 9 ± 0.3 months for Alentejana and Barrosã young bulls, respectively. At the start of experiment, the average live weight was 266 ± 45.8 kg and 213 ± 16.3 kg for Alentejana and Barrosã young bulls, respectively. The experiment lasted from January to November 2009. One Alentejana bull from the HS dietary group was removed from the study due to a limp.

A pre-trial period of three weeks was followed by the finishing period that lasted until each animal reached 18 months of age. Twice daily, bulls were fed with the experimental diets *ad libitum* and water was provided. During the experiment, animals were individually weighed every 14 days, before feeding. Feed offered and refusals were recorded daily to calculate feed intake for each pen. The ratio between weight gain and DM intake was used to calculate the feed efficiency *per* pen. The DM intake and feed efficiency were calculated for the period during which each pen was complete (five animals).

Table 2.1 – Ingredients, chemical and fatty acid composition of the high (HS) and low silage (LS) diets (*n*=3)

	HS	LS	Concentrate feed
Ingredients (%)[†]			
Maize silage	70	30	
Concentrate feed	30	70	
Ingredients (%)[ǂ]			
Maize			32.5
Wheat			20.1
Barley			19.7
Soybean meal			13.5
Sunflower meal			8.0
Hydrogenated fat			1.3
Calcium carbonate			2.0
Sodium bicarbonate			1.0
Calcium phosphate			0.9
Salt			0.8
Vitamin premix			0.2
Chemical composition (unit/kg DM)			
Crude protein (g)	142	125	
Crude fat (g)	28.7	31.7	
Crude fibre (g)	198	150	
NDF (g)[§]	403	321	
ADF (g)[§§]	249	186	
Ash (g)	55.3	61.7	
Starch (g)	285	376	
Gross energy (MJ)	19.1	18.6	
Fatty acid composition (mg/g DM)			
16:0	16.1	16.5	
18:0	4.05	6.33	
*c*9-18:1	12.1	11.0	
18:2*n*-6	35.1	28.2	
20:0	5.22	2.61	
18:3*n*-3	7.36	4.21	
Total fatty acids	80.0	68.9	

[†]dry matter basis.
[ǂ]fresh weight basis
[§]NDF = neutral detergent fibre.
[§§]ADF = acid detergent fibre

2.2.2 Slaughter and sampling procedures

2.2.2.1 Carcass measurements

Each animal was slaughtered when reached 18 months of age, at the INIAV Experimental Abattoir, by exsanguination after stunning with a cartridge-fired captive bolt stunner. Cod, kidney knob and channel fat (KKCF), mesenteric and omental fat depots were excised and weighted. The carcasses were split along the column, half carcasses weights were recorded and dressing percentage calculated from the ratio between slaughter weight (SW) and hot carcass weight (HCW). Muscle pH and temperature were measured at 45 minutes, 3 and 24 hours after slaughter using a pH meter equipped with a penetrating electrode (Hanna Instruments, HI8424, Smithfield, RI, USA), at the *longissimus thoracis* (LT) muscle between the 12th and 13th ribs. Carcass conformation and carcass fatness scores were determined according to the EUROP classification (Commission of the European Communities, 1982) on a continuous 15 point scale, as described by Hickey, Keane, Kenny, Cromie and Veerkamp (2007).

Carcasses were suspended from the Achilles tendon, weighed, chilled at 10 ºC for 24 hours and submitted to an ageing period of 8 days at 2 °C. The left half carcass was subsequently separated into commercial joints and the leg joint was dissected into the component tissues: muscle, intermuscular and subcutaneous fats, and bone. This joint has been suggested to be representative of the overall bovine carcass composition, at least, in these particular breeds (Simões & Mendes, 2003).

After the ageing period, the LL and *semitendinosus* (ST) muscles were removed from the carcasses. The muscles investigated were chosen due to their divergent growth patterns and functions. In addition, they represent meat cuts of different expected quality and economical value. For shear force and cooking loss measurements, LL (1^{st} to 3^{rd} lumbar vertebra) and ST muscles were sliced into 2.5-cm-thick steaks. For lipid content analysis, both muscles (4^{th} to 6^{th} lumbar vertebrae, LL; medium portion, ST) were trimmed of connective and adipose tissues, and blended in a food processor. All samples were vacuum packed and stored at −20 ºC until further analysis.

2.2.2.2 Meat colour

The colour of the LT muscle (13^{th} thoracic vertebra), after the aforementioned ageing period, was measured using a Minolta CR-300 chromometer (Konica Minolta, Portugal) in the L*, a* and b* system, one hour after air exposure to allow blooming. Hue and chroma values were

calculated as arctg(b / a) and $\sqrt{(a^2+b^2)}$, respectively (Dunne, Keane, O'Mara, Monahan, & Moloney, 2004). Enhanced redness (E*) was calculated using the following modified equation as described by Liu, Fan, Chen and Thayer (2003): E* = a/b+a/L.

2.2.2.3 Lipid content

Meat samples (LL and ST muscles) were lyophilized (−60 ºC and 2.0 hPa) to constant weight, using a lyophilisator (Edwards High Vacuum International, West Sussex, UK), kept dry at room temperature and analysed within two weeks. For IMF determination, total lipids were extracted from lyophilized meat samples (*ca*. 250 mg) by the method described by Folch, Lees and Stanley (1957), using dichloromethane and methanol (2:1 v/v) instead of chloroform and methanol (2:1 v/v), as described by Carlson (1985). Total lipids were measured gravimetrically, in duplicate, by weighing the fatty residue obtained after solvent evaporation.

2.2.2.4 Shear force and cooking loss

Steaks of LL muscle were thawed at room temperature and cooked in a plate grill (65/70 FTES Electric Griddle, Modular Catering Equipment, Italy) at 250 °C, until the internal thermocouple (Lufft C120, München, Germany) reached 71 °C. All cuts were weighed before and after cooking to determine the cooking loss. After cooling for one hour, 8−10 cores parallel to muscle fibre direction, with 1 cm^2, were taken from each steak. A texture analyser (TA-tx2i Texture Analyzer, Stable Micro Systems, Godalming, UK) equipped with a Warner-Bratzler shear device was used to measure shear force (kg). Specific software was used for data collection (Texture Expert Exceed, Stable Micro Systems).

2.2.2.5 Statistical analysis

For statistical analyses, the SAS software version 9.2 (SAS Institute Inc., 2009) was used. All statistical analyses were performed based on a 2 × 2 factorial arrangement of breed (Alentejana and Barrosã purebreds), diet (HS and LS diets) and their respective interaction. The pen was the experimental unit for DM intake and feed efficiency. For the remaining parameters, the individual animal was considered the experimental unit.

The variances were tested for heteroscedasticity and, for most parameters, variance was found to be heterogeneous. Therefore, subsequent data analysis was performed in order to account for heterogeneous variance. The general Satterthwaite approximation was computed in a mixed-effects regression model (PROC MIXED; SAS Institute Inc., 2009), with breed, diet and their interaction as fixed effects.

The relationships among adipose tissue depots, leg joint composition and carcass traits were tested using the CORR procedure of SAS (SAS Institute Inc., 2009).

Data were expressed as mean ± standard error. Statistical significance was considered when $P<0.05$ and a trend towards significance was assumed when $0.10>P>0.05$ for all the statistical analyses performed.

2.3. Results and discussion

2.3.1 Productive traits

Results concerning animal performance are depicted on Table 2.2. The DM intake was higher in Alentejana in comparison to Barrosã bulls and, in turn, average daily gain (ADG) was lower in the bulls from the latter breed ($P<0.05$ and $P<0.001$, respectively). This could be a consequence of their different genetic potential for growth (Silva *et al.*, 1998), as well as their DM intake capacity. In addition, it is known that, for some beef breeds, gut and digestive system capacity can limit voluntary feed intake and, therefore, reduce live weight gain (Albertí *et al.*, 2008). In general, feeding animals with forage-based diets results in lower growth rates than those obtained with concentrate-based diets (Steen & Kilpatrick, 2000; Nuernberg *et al.*, 2005), because the latter provides more net energy to be partitioned to growth and maintenance. In our study, LS-fed animals had slightly higher ADG than those fed the HS diet, although the difference failed to reach statistical significance. The use of maize silage in this experiment, instead of a forage with low energy value (such has grass silage), might explain why the differences on ADG between diets were not as expressive as reported by other authors. Similarly, there were no variations among the four experimental groups for feed efficiency ($P>0.10$).

As expected, the SW was higher in Alentejana when compared to Barrosã bulls ($P<0.001$). Alentejana (large-framed) and Barrosã (small-framed) bovines are quite distinct regarding their mature size (Silva *et al.*, 1998). Diet had no influence on SW ($P>0.05$), in spite of the highest values observed for the LS-fed bulls.

2.3.2 Carcass traits

The effect of breed was noticeable on HCW, with Alentejana bulls producing heavier carcasses than Barrosã bulls (*P*<0.001) (Table 2.2). On the other hand, diet had no impact on HCW (P>0.10). This observation is in agreement with the reports by French *et al.* (2001), who reported similar carcass weights when diets with different grass to concentrate ratios were provided to beef cattle. Carcasses from Alentejana bulls had higher dressing percentage than those from Barrosã bulls (*P*<0.05). Usually, dressing percentage is related to finishing degree, amount of visceral fat and gut fill (Simões, Mira, Lemos, & Mendes, 2005). Jones, Rompala and Jeremiah (1985) suggested that visceral fat could partially account for the differences in dressing percentage.

Table 2.2 – Effect of breed and diet on the productive and carcass quality traits from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets

	AL		BA		Significance		
	HS	LS	HS	LS	Breed	Diet	Breed × Diet
Performance traits							
DMI (kg/day)[a]	42.5 ± 1.99	40.8 ± 3.39	29.6 ± 0.39	29.5 ± 0.45	*	ns	ns
ADG (g/day)[b]	1670 ± 71.0	1775 ± 93.1	901 ± 25.7	1033 ± 75.3	***	ns	ns
Feed efficiency (kg/kg)	0.191 ± 0.001	0.218 ± 0.003	0.152 ± 0.002	0.178 ± 0.018	ns	ns	ns
SW (kg)[c]	622 ± 17.7	636 ± 29.7	457 ± 8.88	497 ± 23.0	***	ns	ns
Carcass traits							
HCW (kg)[d]	357 ± 9.7	371 ± 16.8	257 ± 6.2	284 ± 14.5	***	ns	ns
Dressing percentage [e]	57.4 ± 0.65	58.3 ± 0.37	56.3 ± 0.52	57.0 ± 0.70	*	ns	ns
Conformation score [f]	7.11 ± 0.633	6.80 ± 0.533	7.20 ± 0.574	6.60 ± 0.562	ns	ns	ns
Fatness score [g]	5.11 ± 0.200	5.60 ± 0.267	5.40 ± 0.221	6.60 ± 0.340	*	**	ns
Visceral fat (%) [h]	6.33 ± 0.357	6.42 ± 0.337	6.01 ± 0.391	7.69 ± 0.591	ns	*	‡
Cod fat (%)	0.323 ± 0.025	0.265 ± 0.025	0.352 ± 0.027	0.381 ± 0.014	**	ns	‡
Mesenteric fat (%)	1.55 ± 0.103	1.68 ± 0.141	1.52 ± 0.121	2.09 ± 0.173	ns	*	ns
Omental (%)	2.11 ± 0.164	2.41 ± 0.137	1.90 ± 0.117	2.84 ± 0.204	ns	***	‡
KKCF (%)[i]	2.35 ± 0.130	2.07 ± 0.137	2.25 ± 0.197	2.38 ± 0.241	ns	ns	ns

[a] DMI = dry matter intake.
[b] ADG = average daily gain.
[c] SW = slaughter weight.
[d] HCW = hot carcass weight.
[e] Dressing percentage = (HCW×100/SW)
[f] Carcass conformation score: poor (P), fair (O), good (R), very good (U) and excellent (E); scoring from 1 for P^- to 15 for E^+.
[g] Carcass fatness score: low (1), slight (2), average (3), high (4) and very high (5); scoring from 1 for 1^- to 15 for 5^+.
[h] Sum of KKCF, mesenteric, omental and cod fats (% of HCW)
[i] KKCF = kidney knob and channel fat.
Significance: ns, *P*>0.1; ‡, *P*<0.1; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.The symbols used mean as follows: DMI, dry matter intake; ADG, average daily gain; SW, slaughter weight; HCW, hot carcass weight; KKCF, kidney knob and channel fat.

However, the breed differences for cod fat, which represented about 0.3% of the carcass, are certainly insufficient to explain the variations in dressing percentage. The lower dressing percentage could also be related with SW, which is in agreement with the results of Jones *et al.* (1985) and Simões *et al.* (2005). Those authors reported that the alimentary tract and the hide account for 74% of the variation in dressing percentage. In addition, Kempster, Cook and Southgate (1982) reported that large crossbreds had higher dressing percentages than small crossbreds. Moreover, the proportion of muscle should also be considered, as Alentejana bulls tended (P=0,099) to have a slightly higher muscle proportion, in the leg joint, than Barrosã bulls.

Neither breed nor diet influenced the carcass conformation score ($P>0.05$), whereas fatness score was significantly different between breeds and diets, being higher in Barrosã than in Alentejana ($P<0.05$), as well as in LS- than in HS-fed animals ($P<0.01$). Conformation and fatness scores have shown to be reliable predictors of carcass traits. Therefore, meat yield may be estimated from these carcass classification scores (Drennan, McGee & Keane, 2008; Clarke *et al.*, 2009; Conroy, Drennan, Kenny & McGee, 2010). Considering that fatness scores increase with age (Piedrafita *et al.*, 2003), the higher scores obtained for Barrosã carcasses in this study might indicate higher maturity of these bulls, at least from a commercial point of view. Diet was also a source of variation on fatness scores, and the higher starch contents in the LS diet, in comparison to the HS diet, likely promoted carcass backfat deposition.

A better understanding on the regulation of body fat deposition is essential to devise strategies to manipulate fat partitioning and, consequently, improve carcass composition. Our results showed that visceral fat percentage was not different between breeds, whereas both breed and/or diet determined the proportion of the individual visceral fat depots. The proportion of cod fat was higher in Barrosã in comparison to Alentejana animals ($P<0.01$), although no difference was found between feeding strategies ($P>0.05$). Both mesenteric and omental fats were lower ($P<0.05$) in HS- than in LS-fed bulls. The percentages of KKCF were unchanged among experimental groups ($P>0.05$). Diet influenced the proportion of all visceral fat depots, except cod fat, which were consistently higher in LS-fed bovines than in HS-fed animals. The differences observed could be explained by the higher starch and lower fibre contents in the LS diet relative to the HS diet, as these variables affect ruminal fermentation, small intestine digestion and net energy availability. The LS diet promotes higher levels of propionate from ruminal fermentation and/or higher glucose absorption in the small intestine from non-degradable starch than the HS diet (Schoonmaker, Trenkle & Beitz, 2010). This would, in turn, promote insulin production and therefore stimulate lipogenesis (Schoonmaker *et al.*, 2003). According to Laviola *et al.* (2006), the sensitivity of adipocytes to insulin exposure varies according to fat depot location, which may be due to differential β-

adrenoreceptor expression and activity (Giorgino, Laviola, & Eriksson, 2005). This could explain the differential effect of diet influence on the visceral fat depots.

The dissection of the leg joint (Table 2.3) showed that Barrosã bulls had higher proportions of intermuscular and total fat than Alentejana bulls, but lower muscle/bone ratios (P<0.05). Subcutaneous fat in the leg joint tended (P=0.070) to be higher in the Barrosã when compared to Alentejana, whereas the muscle proportion showed the inverse trend (P=0.099). In addition, bovines fed the LS diet tended (P=0.057) to show higher subcutaneous fat deposition in leg joint than animals fed the HS diet. Usually, large cattle breeds tend to take longer time to reach the same total fat proportion in the carcass than small breeds (Simões & Mira, 2002). Barrosã breed has a lower mature weight than Alentejana and is relatively early maturing (Silva *et al.*, 1998). In fact, Silva *et al.* (1998) described a preferential deposition of subcutaneous and intermuscular fats in Barrosã animals, as it approaches maturity. It was, therefore, expected that carcasses from early-maturing Barrosã breed bulls would show fatter carcasses than those from Alentejana, when compared at the same age. In agreement with this, higher proportions of subcutaneous and intermuscular fats in the leg joint were found in the Barrosã breed in comparison to Alentejana in our experimental conditions. The same pattern was also observed for fatness scores.

Table 2.3 – Effect of breed and diet on the leg joint dissection variables and intramuscular fat from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets

	AL		BA		Significance		
	HS	LS	HS	LS	Breed	Diet	Breed × Diet
Leg dissection (g/100 g)							
Muscle	76.9 ± 0.561	76.8 ± 0.844	73.8 ± 2.20	75.0 ± 1.44	‡	ns	ns
Bone	13.0 ± 0.26	12.6 ± 0.20	13.2 ± 0.39	13.4 ± 0.60	ns	ns	ns
Subcutaneous fat	4.10 ± 0.162	4.59 ± 0.221	4.54 ± 0.410	5.92 ± 0.765	‡	‡	ns
Intermuscular fat	4.96 ± 0.286	5.01 ± 0.387	5.71 ± 0.640	6.49 ± 0.646	*	ns	ns
Total fat proportion[*]	9.06 ± 0.353	9.60 ± 0.574	10.2 ± 0.839	12.4 ± 1.25	*	ns	ns
Ratios							
Muscle/fat	8.61 ± 0.407	8.22 ± 0.437	7.54 ± 0.538	7.06 ± 1.15	ns	ns	ns
Muscle/bone	5.94 ± 0.120	6.10 ± 0.115	5.62 ± 0.239	5.67 ± 0.187	*	ns	ns
Intramuscular fat							
IMF_{LL} (mg/g muscle) [§]	12.1 ± 0.76^{c}	12.5 ± 0.84^{c}	17.6 ± 1.21^{b}	27.6 ± 1.96^{a}	***	***	***
IMF_{ST} (mg/g muscle) [†]	7.15 ± 0.535	12.2 ± 1.69	9.47 ± 1.01	14.6 ± 1.81	‡	***	ns

[*] Sum of subcutaneous and intermuscular fat percentages in the leg.
[§] IMF_{LL}, intramuscular fat in the *longissimus lumborum* (LL) muscle.
[†] IMF_{ST}, intramuscular fat in the *semitendinosus* (ST) muscle.
[a,b,c] Least square means in the same row with different superscripts are significantly different ($P<0.05$); significance: ns, P>0.1; ‡, P<0.1; *, P<0.05; **, P<0.01; ***, P<0.001.

When comparing Alentejana and Barrosã breeds at the same proportion of total fat, Simões and Mendes (2003) reported no significant variations between breeds concerning the subcutaneous fat of the leg joint. According to these authors, any difference found in the subcutaneous fat of the leg would, therefore, reflect discrepancies in the carcass total fat and, consequently, in maturity.

The muscle/bone ratio is little affected by differences in the degree of maturity, and each breed displays a distinctive value over quite a wide range of fatness (Kempster *et al.*, 1982). Our results showed that Barrosã breed had a lower muscle/bone ratio in the leg joint than Alentejana, which could indicate a lower muscle deposition in the carcass of the former breed. On this concern, Jones, Burgess, Wilton and Watson (1984) described that breeds with higher adult weight, display higher muscle/bone ratios, whereas Kempster (1986) reported that a better dressing percentage is an indicator of a higher muscle/bone ratio, which is in agreement with our data. In addition, Piedrafita *et al.* (2003) reported negative correlations between carcass muscle and bone proportions, estimated from the dissection of the rib joint.

2.3.3 Intramuscular fat

Breed and diet are major factors influencing lipid deposition in animals. However, muscle location and, therefore, function may also account for a differential IMF deposition. A diet and a breed×diet interaction effect was obtained for IMF content in ST and LL muscles, respectively (P<0.001) (Table 2.3). The IMF content in LL muscle was higher in LS-fed than in HS-fed Barrosã bulls, although a similar effect was not observed for the Alentejana bulls. The breed×diet interaction observed for IMF content in the LL muscle might be due to genetic differences in fat deposition between bovine breeds. Typically, meat from concentrate-fed cattle has higher IMF contents than those from animals fed on silage or pasture (Wood *et al.*, 2008). Regarding the ST muscle, IMF was higher in the LS-fed than in the HS-fed animals (P<0.001). In addition, the IMF content in the ST muscle tended (P=0.0964) to be higher in Barrosã when compared to Alentejana bulls. Therefore, results reported herein suggested that the response to the diet is not only breed-related but also muscle-specific. Similarly, Jurie *et al.* (2007), in a study comparing distinct muscle types, found higher TAG contents in the LT than in the ST muscle.

2.3.4 Meat quality

The rate of temperature decline is of extreme importance. In fact, the temperature at 2.5 hours *post-mortem* has shown direct and indirect effects on meat tenderness (Lochner,

Kauffman, & Marsh, 1980; May, Dolezal, Gill, Ray, & Buchanan, 1992). Moreover, muscle pH and temperature interact during *rigor mortis* development, impacting on both physical shortening and proteolytic enzyme activity. (Dransfield, Etherington & Taylor, 1992; Tornberg *et al.*, 2000). Both pH and temperature at 45 minutes and 3 hours *post-mortem* were higher in Alentejana than in Barrosã bulls (at least, $P<0.05$) (Table 2.4). However, similar values were observed at 24 hours after slaughter ($P>0.05$). Pasture-raised animals usually have higher muscle ultimate pH than animals fed on concentrate (Sheath, Coulon, & Young, 2001). Additionally, ultimate pH variability is wider and the glycolytic potential is lower in animals fed on pasture than in those fed on concentrate (Young, Daly, Graafhuis, & Moorhead, 1997; Vestergard, Oksbjerg, & Henckel, 2000). Our data showed that pH was not influenced by diet at 45 minutes and 3 hours *post-mortem*, but it should be taken into account that maize silage does not mimic a pasture-based diet. In contrast, breed determined the initial pH value. These results may reflect a distinct fibre profile between breeds, which would in turn determine a differential glycolytic potential responsible for a faster decrease in the LL muscle pH of the Barrosã breed (Anderson, Lonergan, Fedler, Prusa, Binning & Huff-Lonergan, 2012).

Concerning the colour-related parameters, higher L^* values were observed in Alentejana than in Barrosã meats, which was reflected on the Hue value ($P<0.01$) (Table 2.4). No variations among experimental groups were found for Chroma, whereas E^* tended to be lower in Alentejana than in Barrosã meat ($P=0.056$). Several factors can influence colour, including diet and ultimate pH (Priolo *et al.*, 2001). Our results showed that diet had no significant effect on colour variables, which is consistent with the findings reported by French *et al.* (2001). The inverse correlation between muscle ultimate pH and meat lightness has long been established (Renerre, 1981). Nevertheless, other authors have found that pH is not a major determinant of colour stability over a very limited range, 5.6–5.8 (Young & Kauffman, 1978; Baublits *et al.*, 2004). Direct effects of diet on meat colour seldom occur and depend on the diet ability to influence muscle myoglobin content (Priolo *et al.*, 2001). Muscle colour may also reflect IMF content (Muir *et al.*, 1998), as well as muscle fibre type composition (Lafaucheur, 2010), all of which change according to age and growth rate.

Literature findings on the effects of SW and fatness on meat colour are contradictory. Campion, Keane, Kenny, and Berry (2009) reported a decrease in both L* and hue as SW increased, whereas others (Dunne *et al.*, 2004) have found that a relationship for bulls but not for steers. According to Simões (2006) low L* values correspond to high IMF contents, which is in agreement with a higher deposition of IMF in Barrosã bulls. Simões (2006) reported a decrease in L values and a raise in the a/b ratio as growth proceeds, leading to progressively darker meats. Lightness is also influenced by precociousness as higher myoglobin content and consequently lower L* values are usually associated to more

precocious animals (Renerre, 1981). Finally, higher growth rates, as observed in this study for Alentejana breed, involve a lower deposition of the heme pigment contributing to a lighter meat (Piedrafita *et al.*, 2003). Moreover, it is usually observed a shift towards the production of fibres with greater glycolytic activity in animals with high weight gain, thus leading to a faster *post-mortem* maturation rate (Piedrafita *et al.*, 2003).

Table 2.4 – Effect of breed and diet on the *longissimus* muscle quality traits from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets

	AL		BA		Significance		
	HS	LS	HS	LS	Breed	Diet	Breed × Diet
Temperature							
45 m	37.6 ± 1.11	36.2 ± 1.54	29.8 ± 0.29	29.0 ± 0.59	***	ns	ns
3 h	26.7 ± 1.46	27.9 ± 0.95	24.2 ± 0.79	25.3 ± 0.83	*	ns	ns
24 h	15.2 ± 1.06	15.6 ± 1.06	15.6 ± 0.79	15.0 ± 0.53	ns	ns	ns
pH							
45 m	6.79 ± 0.072	6.75 ± 0.069	6.52 ± 0.062	6.40 ± 0.066	***	ns	ns
3 h	6.45 ± 0.071	6.24 ± 0.073	6.16 ± 0.059	6.20 ± 0.065	*	ns	‡
24 h	5.65 ± 0.060	5.71 ± 0.077	5.73 ± 0.067	5.67 ± 0.052	ns	ns	ns
Colour							
L^*	38.8 ± 0.78	38.9 ± 0.818	37.5 ± 0.568	35.8 ± 0.76	**	ns	ns
a^*	17.7 ± 0.51	16.2 ± 1.24	17.6 ± 0.46	19.0 ± 1.02	ns	ns	ns
b^*	8.79 ± 0.425	7.47 ± 0.834	7.72 ± 0.522	7.88 ± 0.514	ns	ns	ns
Hue	26.4 ± 0.75	24.0 ± 1.47	23.4 ± 0.95	22.4 ± 0.68	*	ns	ns
Chroma	17.9 ± 1.44	19.8 ± 0.61	20.6 ± 1.12	19.2 ± 0.62	ns	ns	ns
E^*	2.49 ± 0.078	2.79 ± 0.200	2.82 ± 0.110	2.98 ± 0.095	‡	‡	ns
Shear force[§]							
WBSF (kg)[†]	6.37 ± 0.732	6.32 ± 0.389	4.83 ± 0.361	5.20 ± 0.544	*	ns	ns
Cooking loss (%)	29.4 ± 2.61	29.5 ± 1.71	34.5 ± 1.44	31.4 ± 1.23	‡	ns	ns

Significance: ns, $P>0.1$; ‡, $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
[§] Published in Costa *et al.* (2012).
[†] WBSF, Warner-Bratzler shear force.

Alentejana meat had higher values of shear force than Barrosã meat (P<0.05) (Table 2.4). Conversely, the cooking loss percentage tended to be higher in Barrosã meat (P=0.067). It is well known that cooking loss depends on the properties of raw meat, namely moisture, fat, protein composition and pH (Del Campo, Brito, Lima, Hernández, & Montossi, 2010). In the present study, the higher WBSF values found for the LL muscle of the Alentejana breed are in agreement with its lower IMF content. Usually, a curve–linear relationship between tenderness and final pH is observed (Purchas *et al.*, 1999). Moreover, the rate of pH decline

has a major influence meat tenderness, with a rapid fall being associated with meat with lower WBSF values (O'Halloran, Troy & Buckley, 1997; Tornberg, Wahlgren, Brondum & Engelsen, 2000; Rosenvold, Andersen, Slinde & Hildrum, 2003) In fact, the LL muscle pH was consistently lower in the Barrosã than in the Alentejana breed carcasses during the first three hours *post-mortem*.

2.3.5 Correlation analysis

The correlation analysis (Table 2.5) showed an association between the subcutaneous and mesenteric fats in the Alentejana bulls, indicating a parallel deposition of these depots.

Table 2.5 – Pearson's correlations among carcass traits of Alentejana and Barrosã bulls

Alentejana	SCFL	interMF	KKCF	Mesenteric	Omental	IMF_{LL}	IMF_{ST}
SCFL	1.00						
interMF	0.44	1.00					
KKCF	0.01	0.10	1.00				
Mesenteric	0.46*	0.27	0.45	1.00			
Omental	0.45	0.41	0.68**	0.78***	1.00		
IMF_{LL}	0.21	0.09	-0.11	0.44	0.21	1.00	
IMF_{ST}	0.26	-0.06	0.23	0.42	0.43	0.03	1.00
Barrosã							
SCFL	1.00						
interMF	0.47*	1.00					
KKCF	0.63**	0.16	1.00				
Mesenteric	0.75***	0.18	0.84***	1.00			
Omental	0.74***	0.29	0.84***	0.95***	1.00		
IMF_{LL}	0.68**	0.54*	0.47*	0.72***	0.76***	1.00	
IMF_{ST}	0.25	0.22	0.55**	0.54*	0.60**	0.44	1.00

Significance: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
The symbols used mean as follows: SCFL, subcutaneous fat in the leg joint; InterMF, intermuscular fat; KKCF, kidney knob and channel fat; IMF_{LL}, intramuscular fat in the *longissimus lumborum* (LL) muscle; IMF_{ST}, intramuscular fat in the *semintendinosus* (ST) muscle.

A close association was observed between some visceral fats (KKCF and omental; mesenteric and omental). Concerning the Barrosã breed, the correlation coefficients showed close relationships between subcutaneous and KKCF, mesenteric, omental and the IMF in the LL muscle. Linear relationships for the IMF content in LL muscle and the weights of subcutaneous, mesenteric, omental and perirenal adipose tissues were described for

Japanese Black and European beef cattle breeds (Belgium Blue, German Angus, Holstein Friesian) (Gotoh *et al.*, 2009). However, in the present study, no significant correlation between the IMF from both muscles and each of the visceral fat depots of the Alentejana bulls were found. In contrast, significant correlations between the IMF from LL muscle and subcutaneous, mesenteric and omental fats were found for Barrosã bulls. Similarly, significant correlations between IMF content in ST muscle and KKCF, mesenteric and omental fats were also observed. These results suggest that, in the Alentejana bulls, only mesenteric fat was being deposited along with subcutaneous fat, which is in line with the data obtained by Silva *et al.* (1998). Those authors who reported that, as it reaches maturity, Alentejana breed deposits mainly internal fat. The present study also suggests a higher deposition of visceral and subcutaneous fats in parallel to IMF in Barrosã bulls. In contrast, the fact that no strong correlation between IMF and the remaining fat depots was found for the Alentejana breed could indicate that feeding strategies to increase IMF, without an increase of "waste fat", would be possible.

2.4. Conclusions

Overall, the results presented here showed that, under these experimental conditions, genetic background is the most prominent factor in carcass fat partitioning. Moreover, the results indicate strong correlations between the IMF content and most of the fat depots in Barrosã bulls. However, the same associations were not found for the Alentejana breed. This suggests that these breeds have different fat deposition patterns, which could imply that an increase in IMF deposition in Alentejana bulls would be possible without the undesirable accumulation of visceral fat. Nonetheless, further studies are needed for the full understanding of the factors and mechanisms underlying lipid metabolism and, thus, carcass composition and meat quality.

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CHAPTER 3

Cellularity and lipid profile of subcutaneous and mesenteric adipose tissues

Contrasting cellularity and fatty acid composition in fat depots from Alentejana and Barrosã bovine breeds fed high and low forage diets

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ASH Costa collaborated in the sample collection, determined the fatty acid content and composition of samples, and performed the associated data processing and statistical analysis. In addition, ASH Costa participated in the interpretation and discussion of the results, as well as in the writing the manuscript.

Abstract

During the finishing phase of bovines, large amounts of subcutaneous and visceral fats are deposited leading to production inefficiencies with major impact on meat quality. A better understanding of the cellularity features of the main fat depots could provide strategies for adipose tissue manipulation. This study assessed the effect of feeding diets with distinct forage to concentrate ratios on the cellularity of two fat depots of beef cattle and their implications on the fatty acid profile. Thus, two phylogenetically distant Portuguese bovine breeds, Alentejana and Barrosã, were selected. The results did not show differences in subcutaneous fat deposition nor in visceral fat depots partitioning. Plasma adipokines concentration failed to show a consistent relationship with fatness, as leptin remained constant in all experimental groups, whereas interleukin-6 was influenced by breed. Fat

depot seems to determine the area and number of adipocytes, with larger adipocytes and a lower number of cells in subcutaneous fat than in mesenteric fat. Neither breed nor diet influenced adipocytes area and number. The contents of total fatty acids, partial sums of fatty acids and conjugated linoleic acid isomeric profile were affected by breed and fat depot. The incorporation of SFA, TFA, PUFA and BCFA was higher in mesenteric fat depot, whereas subcutaneous fat depot had greater percentages of MUFA. In addition, SFA and MUFA proportions seem to be breed-related. In spite of the less relevant role of diet, the percentages of PUFA and BCFA were influenced by this factor. Under these experimental conditions, the effect of fat depot on cellularity and fatty acid composition prevails over breed or diet, as reinforced by the principal component analysis.

3.1 Background

The manipulation of fat deposition in beef cattle is of major importance for the improvement of production efficiency, carcass composition and meat quality. In fact, subcutaneous and visceral fat depots are often not appreciated and, therefore, considered as “waste fat”, whereas intramuscular fat is valued and regarded as “taste fat” (Aldai *et al.*, 2007). Thus, the development of strategies to manipulate adipose tissue deposition in farm animals has been one of the major breeding goals for many years (De Smet *et al.*, 2004).

White adipose tissue, formerly regarded as a passive lipid storage site, is now recognized as a dynamic tissue (Trayhurn & Wood, 2005). It participates in general metabolism by providing substrate for the energy-consuming processes of almost all tissues. The metabolic activity of adipocytes in bovines is under the influence of several factors, namely breed, diet and fat depot location (Hood, 1982). In addition, adipocytes are connected to the vascular network and display an important endocrine role. As developing pre-adipocytes differentiate into mature adipocytes, they acquire the ability to secrete various proteins (Fain, 2006), collectively known as adipokines, like leptin and IL-6. Leptin is an offensive cytokine that controls food intake and energy expenditure, thus regulating feeding behaviour (Vendrell *et al.*, 2004). It also mitigates insulin resistance by stimulating beta-oxidation of fatty acids in the skeletal muscle (Vendrell *et al.*, 2004). IL-6 has a pro-inflammatory activity associated with obesity, impaired glucose tolerance and insulin resistance (Trayhurn & Wood, 2005).

Different metabolic properties, including the regulation of lipid deposition, have been reported in several species for adipocytes of distinct anatomical locations (Hishikawa *et al.*, 2005). Fatty acids of adipocytes derive from *de novo* synthesis or from diet. In cattle, the finishing system can produce important changes in fat deposition, thus suggesting that enzymes involved in lipogenesis are sensitive to dietary energy level and, possibly, to energy source. In fact, fat deposition is determined by the balance between lipogenesis and lipolysis.

Lipogenesis is a process stimulated by a high carbohydrate diet but inhibited by PUFA intake and fasting (Geay *et al.*, 2001). Apart from the amount of fat (Wood *et al.*, 2008), the fatty acid composition, including CLA isomers, of adipose tissue lipids is affected by dietary regimens and breed (Dannenberger *et al.*, 2005).

There is a breed-related pattern of fat deposition during bovines´ growth (Silva *et al.*, 1998). However, the information available on the effect of genetic background on adipose tissue cellularity and fatty acid composition is scarce. Thus, further studies in this field are needed. Genetic distances have been described for some Portuguese autochthonous bovine breeds, independently of their geographical location (Beja-Pereira, 2003). Alentejana is a large bovine breed (Reis *et al.*, 2001) usually reared on a traditional semi-extensive production system in the Southern plains of Portugal (Alfaia *et al.*, 2006). It is the most important commercial PDO-beef (Gabinete de Planeamento e Políticas, GPP, 2009). In contrast, Barrosã is a small breed (Reis *et al.*, 2001) typically reared on a traditional production system in the mountainous Norwest of Portugal (Alfaia *et al.*, 2007), being the most consumed PDO-veal in Portugal (Gabinete de Planeamento e Políticas, GPP, 2009). In addition, large differences in the lipid composition and nutritional quality of intramuscular fat from Alentejana (Alfaia *et al.*, 2009) and Barrosã (Alfaia *et al.*, 2007) bovine meats have been described by our research group.

This experiment was designed to study the effect of breed and diet on cellularity and fatty acid biosynthesis of subcutaneous and mesenteric fat depots from young bulls. For this purpose, two phylogenetically distant autochthonous bovine breeds (Alentejana and Barrosã) and two experimental diets (based on 30/70% and 70/30% of silage and concentrate, respectively) were selected. We hypothesized that: i) the genetic background can determine the bovine fat deposition and partitioning; ii) rearing cattle on different silage/concentrate ratios can alter the fatty acid composition of adipose tissues; iii) the lipid deposition may vary according to the fat depot considered. To achieve these aims, adipocytes size and number (per area) of subcutaneous and mesenteric fat depots were evaluated, through histometrical analysis, in parallel with plasma determination of some adipokines (leptin and IL-6). To further characterize these effects upon cellularity of subcutaneous and mesenteric fats, the detailed fatty acid composition, including the CLA isomeric profile, was determined in both fat depots.

3.2 Material and Methods

3.2.1 Animals and experimental design

Details concerning the experimental design, animals and the composition of experimental diets are provided in Chapter 2, section 2.2.

3.2.2 Sample collection

One week prior to slaughter, blood samples were collected from the tail vein and centrifuged (3000 rpm for 15 minutes at room temperature) to harvest heparinized plasma. The plasma was analysed for some biochemical parameters within 24 hours at a Clinical Chemistry Laboratory (Clínica Médica e Diagnóstico Dr. Joaquim Chaves, Algés, Portugal). All animals were slaughtered at 18 months-old, which is the commercial slaughter age for young bulls in Portugal, at the INIAV experimental abattoir by exsanguination after stunning with a cartridge-fired captive bolt stunner. Mesenteric, omental and kidney knob and channel fat (KKCF) depots were excised and weighed. Subcutaneous adipose tissue was sampled and its amount was determined by dissection of the leg joint. The former has been suggested to be representative of the overall bovine carcass composition, at least in these particular breeds (Simões & Mendes, 2003). For histometrical analyses, samples from subcutaneous and mesenteric fat depots (approximately 100 mg) were fixed by immersion in 10% neutral buffered formalin (Merck, Darmstadt, Germany) for 24 hours and processed for paraffin (Microscopy Histosec, Merck) embedding. A second aliquot from each fat depot was vacuum-packed and stored at –80 ºC until lipid extraction and determination of fatty acid composition and CLA isomeric profile.

3.2.3 Plasma metabolites and adipokines determination

TAG (GPO-PAP) and glucose (GOD-PAP) levels were determined in plasma through diagnostic test kits (Roche Diagnostics, Mannheim, Germany) using a Modular Hitachi Analytical System (Roche Diagnostics). Plasma insulin was quantified using a Bovine ELISA kit (Mercodia, Uppsala, Sweden), leptin through a Multi-Species RIA kit (Linco Research, Millipore, Missouri, USA) and IL-6 using a Bovine ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, Hubei Province, China).

3.2.4 Histometrical analysis

Adipose tissue sections with 10 μm thick were cut on a microtome (Leica, SM 2000R, Nussloch, Germany) from each of the paraffin-embedded specimens. Sections were stained with the classical haematoxylin (Bio-optica, Milan, Italy) and eosin procedure (Richard-Allan Scientific, Kalamazoo, MI, USA) to assess morphology under a light microscope (Olympus BX51 equipped with a DP11 microscope digital camera system, Olympus, Tokyo, Japan). For morphometric analysis, the area (μm^2) of 100 adipocytes from 5 fields per section was determined under the microscope (magnification of ×100), using the DP software for image analysis (Olympus DP-Soft version 3.0 for Windows 95/98). The number of adipocytes was also determined in a fixed area of $\sim 560 \times 10^3$ μm^2 per section (magnification of ×100). The entire histological plan was followed as described by Corino *et al.* (2005).

3.2.5 Fatty acid composition

Subcutaneous and mesenteric fat samples were lyophilised (−60 °C and 2.0 hPa) and maintained at -20 °C until further analysis. Total lipids were extracted by the method of Folch *et al.* (1957), using dichloromethane and methanol (2:1 v/v) instead of chloroform and methanol (2:1 v/v), as modified by Carlson (1985). Fatty acids were converted to methyl esters as described by Raes *et al.* (2001), using sodium methoxide in anhydrous methanol (0.5 mol/l) for 30 min, followed by hydrochloric acid in methanol (1:1 v/v) for 10 min at 50 ºC. Fatty acid methyl esters (FAME) were extracted twice with 3 ml of *n*-hexane and pooled extracts were evaporated at 35 ºC, under a stream of nitrogen, until a final volume of 2 ml. The resulting FAME were then analysed by gas-liquid chromatography using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d., 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), equipped with a flame ionization detector, as described by Bessa *et al.* (2007). The quantification of FAME used nonadecanoic acid (19:0) as the internal standard, added to lipids prior to saponification and methylation. The same FAME solution was used for the analysis of both fatty acid composition and CLA isomeric profile, enabling the direct comparison of quantitative data and eliminating differences in sample preparation. CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids; 250 mm × 4.6 mm i.d., 5 μm particle size; Chrompack, Bridgewater, NJ, USA), using a high performance liquid chromatography (HPLC) system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA) equipped with an autosampler and a diode array detector adjusted to 233 nm, according to the procedure previously reported (Rego *et al.*, 2008). The identification of individual CLA isomers was achieved by comparison of their retention times with commercial and prepared standards, as well as with values

published in the literature. Fatty acid composition was expressed as g/100 g of total fatty acid content, assuming a direct relationship between peak area and fatty acid methyl ester weight.

In order to obtain a detailed profile of conjugated isomers of linoleic acid, CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids; 250 mm × 4.6 mm i.d., 5 μm particle size; Chrompack, Bridgewater, NJ, USA), using a HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA) equipped with an autosampler and a diode array detector adjusted to 233 nm, according to the procedure reported previously by our group (Alfaia *et al.*, 2006). The identification of individual CLA isomers was performed by comparison of their retention times with commercial and prepared standards, as well as with values published in the literature. Commercial standards of individual CLA isomers (*c*9,*t*11, *t*10,*c*12, *c*9,*c*11 and *t*9,*t*11) as methyl esters were acquired from Matreya Inc. (Pleasant Gap, PA, USA). Additional standards of individual (*t*8,*c*10, *c*11,*t*13) and mixtures (*cis,trans*, *trans,cis* and *trans,trans* from 7,9 to 12,14) of CLA isomers were prepared as methyl esters, as described by Destaillats & Angers (2003).

The amounts of CLA isomers were calculated from their silver ion-HPLC chromatogram areas relative to the area of the main isomer *c*9,*t*11 CLA identified by GC (which comprises both *t*7,*c*9 and *t*8,*c*10 CLA isomers). The *c*9,*t*11 co-eluted with isomers *t*7,*c*9 and *t*8,*c*10 in the GC analysis. Therefore, HPLC areas for *t7*,*c9* + *t*8,*c*10+ *c*9,*t*11 were added and used for calculation compared with the three isomer peaks from the GC chromatogram for each sample. The amounts of the other CLA isomers were calculated from their Ag+-HPLC areas relative to the area of the main isomer *c*9,*t*11, as described by Rego *et al.* (2008).

3.2.6 Statistical analysis

Values are presented as mean ± standard error of the mean (SEM) for data concerning growth performance parameters, plasma metabolites and histological analysis. Data analysis was performed using the SAS software package, v9.1 (2004).The effect of breed and diet as main factors, and their interaction (breed×diet), on the body composition and plasma biochemical parameters were analysed by the General Linear Model to perform a two-way analysis of variance. Regarding the analysis of histometrical data, the Sturges' rule (1926) was applied to define the number of classes. The analysis of variance on histometrical data and fatty acid profile was performed using the mixed model, considering the animal as a subject and the fat depot as repeated measures, because the two fats were collected from the same animal. Least squares means were determined using the LSMEANS option and compared, when significant (at $P<0.05$), using the probability difference procedure (PDIFF option). Pearson's correlation coefficients were applied to establish possible relationships

between fat depots mass and their respective adipocytes area. The relationships between cellularity and fatty acid composition in both depots were assessed by the principal component analysis (PCA), using the PRINCOMP procedure of SAS. The PCA was applied to a data set of 78 samples and 36 variables to reduce the dimensionality of the data set and to describe the variability of data in two dimensions. The PCA was used to examine the relationship between the cellularity and fatty acid composition variables considered, enabling not only plots of the relationship between the variables but also attempting to explain those relationships. After data normalization, the principal components were considered significant if they contributed more than 5% for the total variance.

3.3 Results

3.3.1 Fatness and diet are not clearly associated with plasma adipokines

The overall characterization of the studied animal groups, concerning body composition parameters, plasma metabolites and adipokines, is presented in Table 3.1. Live slaughter weight was significantly influenced by breed ($P<0.001$), being the values higher in Alentejana than in Barrosã bulls. Similarly, hot carcass and leg joint weights were higher in Alentejana relative to Barrosã bulls ($P<0.001$). The dissection of the leg showed no differences among groups regarding the subcutaneous fat in the leg joint ($P>0.05$). Mesenteric and omental fats, expressed relatively to the hot carcass weight, were higher in low silage fed animals ($P<0.05$ and $P<0.001$, respectively). The sum of perirenal and retroperitoneal fats (KKCF) showed no influence from breed or diet ($P>0.05$).

No significant effects of breed, diet or interaction were observed for the content of plasma TAG ($P>0.05$). The glucose levels in plasma were affected by breed. The values were higher in Alentejana compared to Barrosã bulls ($P<0.05$). Insulin concentration was affected by diet ($P<0.05$), as low silage diets fed to both breeds promoted higher values of this hormone. Plasma IL-6 concentration was affected by breed, with higher levels in Barrosã than in Alentejana bulls ($P<0.05$). Leptin concentration in plasma was kept unchanged among the four experimental groups ($P>0.05$).

Table 3.1 – Body composition parameters, plasma metabolites and adipokines from Alentejana and Barrosã bulls fed high (HS) or low (LS) silage diets

	Alentejana		Barrosã			Significance level		
	HS	LS	HS	LS	SEM	B	D	B×D
Body composition parameters								
Live slaughter weight (kg)	622	636	457	497	22.3	***	ns	ns
Hot carcass weight (kg)	357	371	257	284	13.1	***	ns	ns
Leg joint weight (kg)	46.8	47.8	35.0	36.0	1.65	***	ns	ns
Subcutaneous fat (g/100 g leg)	4.10	4.59	4.54	5.92	0.459	ns	ns	ns
Mesenteric fat (g/kg carcass)	15.5	16.8	15.2	20.9	1.44	ns	*	ns
Omental fat (g/kg carcass)	21.1	24.1	19.0	28.4	1.65	ns	***	ns
KKCF[a] (g/kg carcass)	23.5	20.7	22.5	23.8	1.92	ns	ns	ns
Plasma metabolites and adipokines								
TAG (mg/L)	175	176	170	184	15.8	ns	ns	ns
Glucose (mg/L)	889	885	820	806	31.4	*	ns	ns
Insulin (μg/L)	0.884	1.80	1.28	2.12	0.359	ns	*	ns
Leptin (μg/L)	3.99	3.82	3.89	5.04	0.451	ns	ns	ns
Interleukin-6 (ng/L)	11.2	8.88	18.4	17.8	3.21	*	ns	ns

[a]Kidney knob and channel fat. B = breed; D = diet. Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

3.3.2 Fat depot, but neither breed nor diet, determines cellularity

Data concerning the histometric evaluation of subcutaneous and mesenteric fats as affected by breed, diet and specific fat depot are summarized in Table 3.2. Except for the fat depot effect ($P<0.001$), no other influence from breed, diet or their interaction was observed ($P>0.05$) for adipocytes area and number. In fact, overall subcutaneous fat had larger adipocytes and a lower number of cells than mesenteric fat (6677 *vs* 5584 μm^2 and 78.0 *vs.* 94.0 cells in 560×10^3 μm^2, respectively).

Table 3.2 also displays data regarding adipocytes area distribution, according to breed, diet and fat depot. Based on the Sturges' rule, ten classes of adipocytes area were constructed. This analysis revealed that, for the smaller adipocytes, there were no differences between fat depots ($P>0.05$). The distribution of fat cells was significantly different in classes containing adipocytes ranging from 5400 to 7200 μm^2 and those larger than 10800 μm^2 ($P<0.05$).Breed played a less relevant role on adipocytes filling and its effect was observed only for two classes. Alentejana bulls had a higher frequency of smaller adipocytes (0-1800 μm^2, $P<0.05$) and adipocytes ranging from 14400 to 16200 μm^2 ($P<0.05$).

The main finding of Table 3.2 is that subcutaneous and mesenteric fats were distinct in terms of adipocytes area and number. Following from this result, we decided to perform a statistical analysis for both fat depots separately (Figure 3.1). For the subcutaneous fat, a diet effect (P<0.05) was observed in the class comprising medium adipocytes, ranging from 7200-9000 μm^2. Indeed, consistently lower frequencies of these medium adipocytes were observed in bulls fed on low silage diets. Moreover, the class with adipocytes ranging from 14400 to 16200 μm^2 was influenced by breed (P<0.05) because the relative frequency of larger adipocytes was higher in Barrosã when compared to Alentejana bulls. In relation to the mesenteric fat, some interesting interactions were observed between breed and diet in the 3600-5400 (P<0.05), 5400-7200 (P<0.05) and 7200-9000 μm^2 (P<0.01) classes. Alentejana bulls fed on low silage diet had lower frequencies of these medium adipocytes than their counterparts fed on high silage. The inverse trend was observed for Barrosã bulls.

The Pearson's correlation coefficient between adipose tissue depot weight and adipocytes area was significant for mesenteric fat (r=0.36, P=0.023), in contrast to subcutaneous fat (r=0.27, P=0.097).

Table 3.2 – Effect of breed, diet and fat depot on the adipocyte area (μm^2), number (in 560×10^3 μm^2) and distribution of subcutaneous (Sub) and mesenteric (Mes) fats from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets.

	Alentejana				Barrosã									
	HS		LS		HS		LS			Significance level				
	Sub	Mes	Sub	Mes	Sub	Mes	Sub	Mes	SEM	B	D	FD	B×D	B×D×FD
Adipocytes														
Area	6759	5353	5931	5217	6842	6087	7177	5676	466	ns	ns	***	ns	ns
Number	76.3	94.3	86.9	100	79.0	89.4	70.0	92.1	6.24	ns	ns	***	ns	ns
Class (μm^2)														
0-1800	19.0^{a}	24.2^{abc}	29.2^{bc}	32.9^{c}	20.1^{a}	22.6^{ab}	21.6^{ab}	20.4^{a}	3.04	*	*	ns	*	ns
1800-3600	11.3	12.8	10.7	12.1	11.8	13.6	13.3	14.2	1.15	ns	ns	ns	ns	ns
3600-5400	12.5	14.9	11.2	11.5	11.6	12.0	10.1	13.8	1.12	ns	ns	ns	ns	ns
5400-7200	14.3^{abc}	16.2^{bc}	10.4^{a}	13.0^{ab}	11.6^{a}	13.4^{abc}	10.7^{a}	17.4^{c}	1.58	ns	ns	**	*	ns
7200-9000	13.9^{ad}	11.8^{ac}	12.8^{abcd}	10.1^{c}	14.3^{ad}	10.4^{bc}	12.7^{abc}	14.9^{d}	1.14	ns	ns	ns	ns	*
9000-10800	9.17	10.2	9.31	7.56	10.3	10.3	8.58	9.87	1.09	ns	ns	ns	ns	ns
10800-12600	4.95	7.30	6.73	5.05	7.29	7.19	7.00	4.98	1.06	ns	ns	*	ns	ns
12600-14400	5.40	2.27	5.54	4.25	7.70	3.66	6.44	2.97	0.954	ns	ns	***	ns	ns
14400-16200	2.31	1.28	3.22	2.66	4.54	2.87	4.88	2.12	0.991	*	ns	*	ns	ns
≥ 16200	4.87	1.50	3.62	3.51	4.24	4.31	8.21	3.06	1.90	ns	ns	*	ns	ns

B = breed; D = diet; FD = fat depot. B×FD and D×FD interactions were not significant ($P<0.05$). Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$).

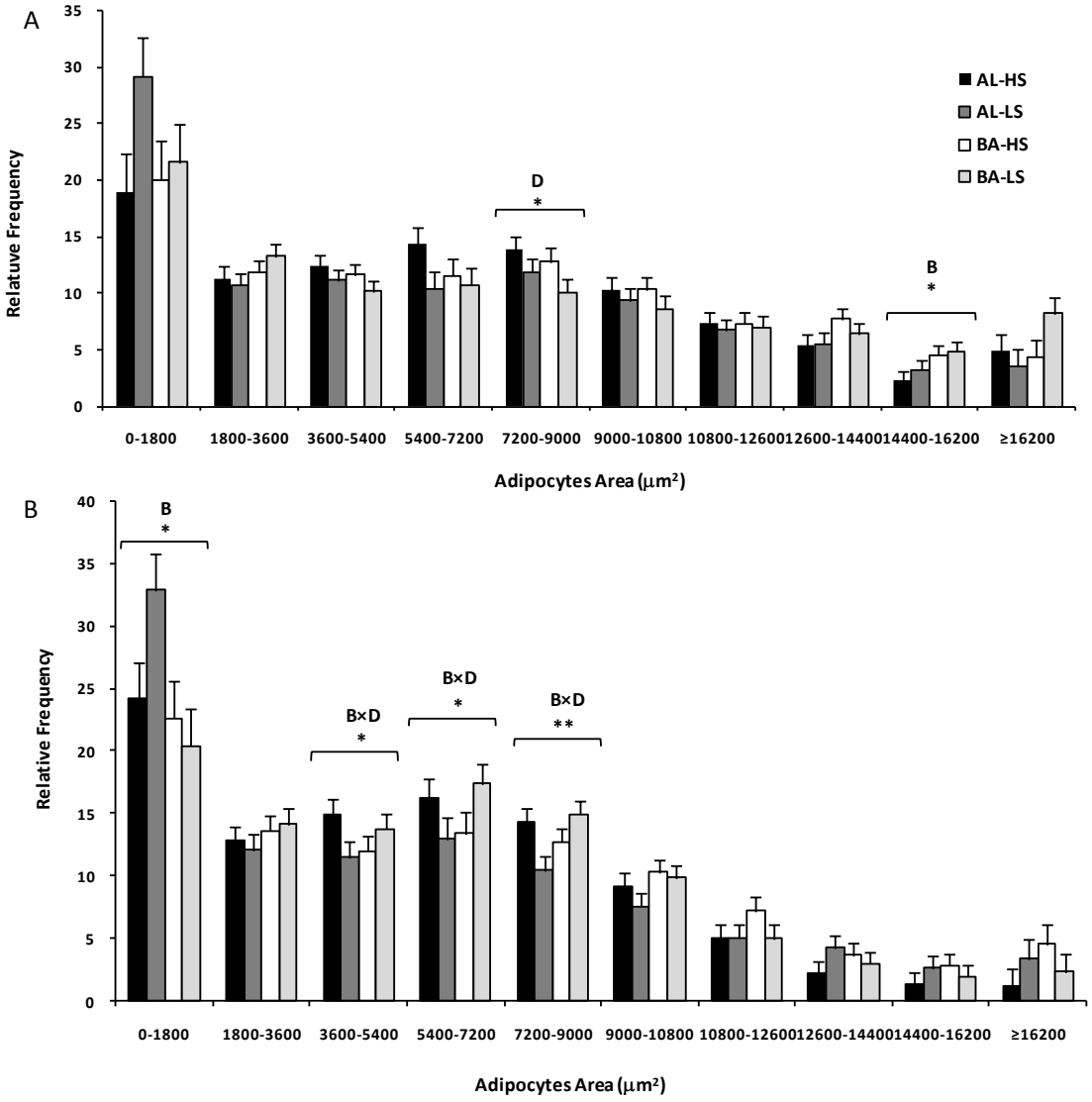

Figure 3.1 – Adipocytes area distribution of subcutaneous (A) and mesenteric (B) fats from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low (LS) silage diets. B = breed; D = diet. Significance level: *, P<0.05; ** P<0.01.

3.3.3 Breed and fat depot are major contributors to a contrasting fatty acid composition

The fatty acid composition of both fat depots from the four experimental groups is depicted in Table 3.3. The predominant SFA were palmitic (16:0) and stearic (18:0) acids, and oleic acid (18:1*c*9) was the main MUFA. In the Alentejana breed the deposition of palmitic acid was higher in subcutaneous than in mesenteric fat depot, whereas the opposite pattern was observed in the Barrosã breed (breed×diet, *P*<0.001). Stearic and oleic fatty acids varied according to breed (*P*<0.001) and fat depot (*P*<0.001) considered. While stearic acid was higher in the mesenteric fat of Alentejana bulls, oleic acid was higher in the subcutaneous fat

of the Barrosã breed. *Trans* octadecenoates (18:1*t*), regarded as the main intermediates arising from C18 PUFA ruminal biohydrogenation, varied according to the main factors. Both breed (P<0.001) and fat depot (P<0.001) determined the proportions of vaccenic acid (18:1*t*11), the main TFA. It was found a greater deposition of this fatty acid in the mesenteric fat from Barrosã breed in comparison to the other experimental groups. The main branched chain fatty acid (BCFA) found in the present study was *a*17:0, a microbial lipid, with the highest percentages observed in the subcutaneous fat of Barrosã bulls fed the high silage diet (P<0.001). Linoleic acid (18:2*n*-6) was the main PUFA, being determined by all main factors (at least P<0.05) and the interaction between breed and diet (P<0.001). The highest proportions of linoleic acid were found in the mesenteric fat of Alentejana bulls fed the low silage diet. A similar pattern was found for 18:3*n*-3, the second most abundant PUFA. Concerning the CLA isomeric profile, significant variations were observed among breeds, diets and fat depots (Table 3.4). The sum of CLA isomers was higher in the subcutaneous fat than in the mesenteric fat, as well as in the Barrosã breed relatively to the Alentejana breed (P<0.001). Individual CLA isomers were mostly influenced by breed and fat depot (at least, P<0.05), although the diet had a strong effect on *t*11,*t*13, *t*11,*c*13 and *t*7,*c*9 isomers (P<0.001), amongst others. The high silage diet promoted greater proportions of *t*11,*t*13, *t*11,*c*13 and *t*12,*t*14, the latter reaching the highest value in the mesenteric fat of Alentejana breed. As far as the *t*10,*c*12 CLA isomer is concerned, a significant deposition was observed in Alentejana bulls fed on low silage diet, regardless the fat depot (P<0.05). Not surprisingly, the predominant CLA isomer in both fat depots was the *c*9,*t*11, which was influenced by breed, diet and fat depot. Its highest value was observed in the subcutaneous fat of Barrosã bulls fed on high silage diet (P<0.001). The proportions of the *t*7,*c*9 CLA isomer were also influenced by the main factors, that is, the highest values were observed in the subcutaneous fat of Alentejana bulls fed low silage diet (P<0.001).

There were significant effects of breed (P<0.001) and fat depot (P<0.001), as well as its respective interaction (P<0.01), on the total SFA and MUFA proportions (Table 3.5). Total TFA were not affected by diet (P>0.05). In contrast, this lipid class was influenced by the fat depot (P<0.001) and its interaction with breed (P<0.05), with higher values in mesenteric fat of Barrosã bulls and subcutaneous fat of Alentejana bulls. Fat depot, diet, and the interactions breed×diet and breed×fat depot, were major determinants on PUFA (at least, P<0.05), with higher percentages observed for low silage fed animals. The high silage diet promoted higher percentages of BCFA in both fat depots (P<0.001). An interaction between breed and fat depot was also observed (P<0.001) due to higher proportions of these fatty acids in the mesenteric fat of Alentejana bulls. Concerning the Δ9-indices (stearoyl-coA desaturase activity indicators), an interaction between breed and diet (P<0.001) was observed, with subcutaneous fat of Barrosã bulls, showing the highest values. The only

Table 3.3 – Total fatty acid content (mg/g fat) and fatty acid composition (g/100 g total fatty acids) of subcutaneous (Sub) and mesenteric (Mes) fats from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana				Barrosã											
	HS		LS		HS		LS			Significance Level						
	Sub	Mes	Sub	Mes	Sub	Mes	Sub	Mes	SEM	B	D	FD	B×D	B×FD	D×FD	B×D×FD
Total fatty acids	496	603	473	558	455	442	436	532	32.5	**	ns	**	ns	ns	ns	ns
Fatty acids																
12:0	0.068^{ac}	0.084^{bc}	0.064^{a}	0.076^{c}	0.065^{ac}	0.103^{d}	0.068^{ab}	0.076^{abc}	0.006	ns	ns	***	ns	*	**	**
*i*14:0	ND	0.115^{a}	ND	0.075^{b}	ND	0.138^{c}	ND	0.068^{c}	0.007	ns	***	***	ns	ns	***	ns
14:0	3.52^{a}	3.23^{ab}	3.50^{a}	3.11^{b}	3.00^{b}	3.52^{a}	3.20^{ab}	3.27^{ab}	0.175	ns	ns	ns	ns	***	ns	ns
14:1*c*9	0.726^{a}	0.138^{b}	0.803^{ac}	0.138^{b}	0.966^{c}	0.166^{b}	1.16^{c}	0.167^{b}	0.072	**	ns	***	ns	**	ns	ns
*i*15:0	0.280^{a}	0.331^{b}	0.141^{c}	0.193^{d}	0.241^{e}	0.318^{ab}	0.157^{cd}	0.187^{d}	0.014	ns	***	***	ns	ns	ns	*
*a*15:0	0.252^{a}	0.461^{b}	0.188^{ad}	0.326^{c}	0.233^{ad}	0.420^{b}	0.176^{d}	0.315^{e}	0.023	ns	***	***	ns	ns	*	ns
15:0	0.429^{ac}	0.686^{b}	0.496^{a}	0.585^{c}	0.456^{ad}	0.711^{b}	0.387^{d}	0.515^{ac}	0.032	ns	*	***	*	ns	***	ns
*i*16:0	0.322^{a}	0.483^{b}	0.182^{de}	0.271^{ae}	0.311^{a}	0.405^{c}	0.171^{d}	0.235^{e}	0.024	ns	***	***	ns	**	***	ns
16:0	27.3^{ad}	25.9^{cd}	26.5^{acd}	25.6^{cd}	23.7^{b}	26.0^{acd}	25.0^{bc}	26.3^{d}	0.559	*	ns	ns	ns	***	ns	ns
16:1*c*7	0.259^{a}	0.398^{c}	0.262^{a}	0.350^{cd}	0.319^{bd}	0.373^{cd}	0.283^{abc}	0.343^{d}	0.016	ns	*	***	ns	***	ns	*
16:1*c*9	4.08^{a}	0.993^{b}	3.78^{a}	1.01^{b}	4.94^{b}	1.04^{b}	5.36^{c}	1.14	0.227	***	ns	***	ns	***	ns	ns
*i*17:0	0.285	0.353	0.24	0.321	0.314	0.382	0.252	0.319	0.014	ns	***	***	ns	ns	ns	ns
*a*17:0	1.31^{a}	1.16^{b}	1.13^{b}	1.01^{c}	1.46^{d}	0.973^{c}	1.35^{a}	0.928^{c}	0.034	ns	***	***	ns	***	ns	ns
17:0	0.920^{a}	1.59^{b}	1.19^{c}	1.65^{b}	0.851^{a}	1.46^{ef}	0.729^{d}	1.28^{cf}	0.041	***	ns	***	***	ns	**	*
17:1*c*9	0.761^{a}	0.381^{b}	1.01^{c}	0.412^{b}	0.947^{c}	0.386^{b}	0.912^{c}	0.383^{b}	0.039	ns	ns	***	*	ns	*	**
*i*18:0	0.243	0.249	0.181	0.187	0.210	0.222	0.148	0.191	0.012	*	***	**	ns	ns	ns	ns
18:0	14.5	32.2	12.9	30.8	11.4	29.3	9.75	27.8	0.859	***	*	***	ns	ns	ns	ns
18:1*t*6-*t*8	0.118	0.187	0.184	0.245	0.136	0.237	0.154	0.231	0.019	ns	*	***	ns	ns	ns	ns

18:1*t*9	0.232	0.252	0.246	0.284	0.240	0.272	0.269	0.289	0.012	ns	*	***	ns	ns	ns	ns
18:1*t*10	0.198^{a}	0.274^{b}	1.04^{c}	0.979^{c}	0.248^{ab}	0.302^{ab}	0.403^{ab}	0.432^{ab}	0.089	**	***	ns	***	ns	*	ns
18:1*t*11	1.41^{ab}	2.21^{c}	1.36^{a}	2.20^{c}	1.88^{bc}	2.90^{d}	1.74^{ab}	2.83^{d}	0.139	***	ns	***	ns	*	ns	ns
18:1*t*12	0.123^{a}	0.297^{cd}	0.202^{b}	0.270^{c}	0.196^{b}	0.338^{d}	0.194^{b}	0.339^{d}	0.021	**	ns	***	ns	ns	ns	*
18:1*c*9	33.4	20.8	34.8	21.7	37.3	22.2	37.3	24.2	0.850	***	ns	***	ns	ns	ns	ns
18:1*c*11	4.13	2.30	3.70	2.51	4.32	2.21	4.53	2.75	0.233	ns	ns	***	ns	ns	ns	ns
18:1*c*12	0.761	0.601	0.721	0.625	0.805	0.629	0.841	0.674	0.036	*	ns	***	ns	ns	ns	ns
18:1*c*13	0.277^{a}	0.111^{b}	0.388^{c}	0.130^{b}	0.405^{c}	0.114^{b}	0.521^{d}	0.145^{b}	0.031	**	**	***	ns	**	*	ns
18:1*t*16+*c*14	0.250^{a}	0.353^{b}	0.198^{c}	0.282^{a}	0.260^{a}	0.328^{b}	0.228^{ac}	0.332^{b}	0.014	ns	**	***	*	ns	ns	ns
18:1*c*15	0.124	0.127	0.141	0.143	0.131	0.106	0.121	0.129	0.009	ns	ns	ns	ns	ns	ns	ns
18:2*c*9,*t*11[§]	0.448^{a}	0.260^{b}	0.500^{a}	0.225^{b}	0.873^{c}	0.439^{a}	0.900^{c}	0.428^{a}	0.034	***	ns	***	ns	***	ns	ns
18:2*t*11,*c*15	ND	0.118^{a}	ND	0.066^{b}	ND	0.132^{a}	ND	0.072^{b}	0.014	ns	**	-	ns	-	-	-
18:2*n*-6	1.49^{a}	1.87^{ce}	2.29^{be}	2.70^{de}	1.70^{ac}	1.94^{c}	1.82^{c}	2.10^{e}	0.104	*	***	***	***	*	ns	ns
18:3*n*-3	0.273^{ac}	0.323^{b}	0.259^{ac}	0.274^{ac}	0.324^{b}	0.379^{d}	0.248^{a}	0.278^{c}	0.012	*	***	***	**	ns	*	ns
19:1	0.254	0.193	0.264	0.186	0.335	0.192	0.301	0.211	0.057	ns	ns	***	ns	ns	ns	ns
20:0	0.093^{a}	0.178^{b}	0.081^{ac}	0.172^{b}	0.079^{a}	0.192^{b}	0.060^{c}	0.170^{b}	0.010	ns	*	***	ns	*	ns	ns
20:1*c*11	0.146	0.096	0.154	0.105	0.169	0.117	0.196	0.133	0.012	**	ns	***	ns	ns	ns	ns
20:3*n*-6	ND	0.062	ND	0.058	ND	0.070	ND	0.066	0.004	ns	ns	-	ns	-	-	-
20:4*n*-6	0.033^{a}	0.045^{abc}	0.034^{ac}	0.058^{b}	0.049^{c}	0.046^{abc}	0.045^{abc}	0.048^{abc}	0.006	ns	ns	*	ns	*	ns	ns

[§]This peak also includes minor amounts of the *t*7,*c*9 and *t*8,*c*10 CLA isomers.

ND = not detected; B = breed; D = diet; FD = fat depot. Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$).

Table 3.4 – CLA isomeric profile (mg/100 g fatty acids) of subcutaneous (Sub) and mesenteric (Mes) fats from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets.

	Alentejana				Barrosã					Significance Level						
	HS		LS		HS		LS									
	Sub	Mes	Sub	Mes	Sub	Mes	Sub	Mes	SEM	B	D	FD	B×D	B×FD	D×FD	B×D×FD
Total CLA	0.516^{a}	0.337^{c}	0.479^{a}	0.285^{c}	0.985^{b}	0.524^{a}	0.992^{b}	0.501^{a}	0.036	***	ns	***	ns	**	ns	ns
*t*12,*t*14	3.17^{ac}	5.77^{d}	2.85^{ac}	4.22^{bc}	4.41^{b}	7.18^{e}	3.56^{c}	6.06^{d}	0.400	***	**	***	ns	ns	*	ns
*t*11,*t*13	6.91^{ab}	15.8^{c}	5.18^{a}	10.9^{d}	9.09^{bd}	20.0^{e}	5.21^{a}	13.2^{f}	0.695	***	***	***	ns	**	***	ns
*t*10,*t*12	4.69^{acd}	5.44^{cd}	7.36^{b}	7.01^{b}	6.48^{bc}	4.57^{d}	6.34^{bc}	6.57^{b}	0.525	ns	**	ns	ns	ns	ns	**
*t*9,*t*11	10.5^{a}	12.5^{a}	10.1^{a}	10.1^{a}	18.6^{bd}	14.6^{cd}	14.3^{abd}	16.9^{d}	2.90	**	ns	ns	ns	ns	ns	*
*t*8,*t*10	3.22^{ac}	5.26^{bc}	3.14^{ac}	3.53^{c}	6.05^{b}	5.25^{b}	5.31^{b}	4.29^{ac}	0.589	**	ns	ns	ns	**	ns	ns
*t*7,*t*9	5.41^{a}	6.22^{ac}	4.42^{ac}	5.23^{ac}	9.59^{b}	5.30^{c}	4.97^{ac}	5.35^{a}	0.670	ns	**	ns	ns	**	**	**
*t*6,*t*8	2.50^{ac}	4.01^{d}	2.02^{a}	1.89^{a}	4.09^{bcd}	3.97^{bd}	3.38^{cd}	1.85^{a}	0.483	ns	***	ns	ns	*	*	ns
total *trans*,*trans*	36.4^{a}	55.0^{bc}	35.1^{a}	42.9^{ac}	64.7^{bc}	60.9^{c}	43.1^{a}	54.2^{b}	4.25	***	**	**	ns	ns	ns	*
c/*t*12,14	2.86^{a}	2.26^{a}	3.31^{ab}	2.11^{a}	4.48^{b}	2.41^{a}	4.40^{b}	2.19^{a}	0.500	*	ns	***	ns	ns	ns	ns
*t*11,*c*13	12.9^{a}	14.6^{d}	5.78^{b}	4.96^{b}	17.9^{c}	17.7^{c}	11.9^{ad}	10.1^{a}	1.05	***	***	ns	ns	ns	**	ns
*c*11,*t*13	3.33^{a}	0.749^{d}	4.19^{ab}	0.429^{d}	5.35^{b}	0.734^{d}	6.97^{c}	0.893^{d}	0.495	***	ns	***	ns	**	*	ns
*t*10,*c*12	7.36^{a}	4.05^{d}	14.9^{b}	10.3^{ac}	11.1^{c}	3.62^{d}	14.5^{b}	5.27^{ad}	1.07	ns	***	***	*	***	ns	ns
*c*9,*t*11	399^{a}	230^{c}	338^{a}	187^{c}	795^{b}	396^{bcd}	806^{b}	377^{ad}	30.8	***	ns	***	ns	***	ns	ns
*t*8,*c*10	8.59^{a}	9.44^{ac}	8.28^{a}	6.54^{a}	15.4^{b}	13.5^{bc}	16.0^{b}	11.9^{c}	0.993	***	ns	**	ns	*	*	ns
*t*7,*c*9	40.9^{ae}	21.3^{d}	63.6^{b}	30.9^{de}	62.7^{b}	29.5^{d}	77.5^{c}	39.1^{e}	3.42	***	***	***	ns	*	*	ns
total *cis*/*trans*	475^{a}	282^{c}	438^{a}	243^{c}	912^{b}	463^{a}	938^{b}	447^{a}	34.6	***	ns	***	ns	***	ns	ns
*c*9,*c*11	5.46^{ad}	2.14^{cd}	6.50^{a}	1.95^{cd}	10.1^{b}	2.86^{cd}	11.1^{b}	3.40^{d}	0.777	***	ns	***	ns	**	ns	ns

D = diet; B = breed; FD = fat depot. Significance level: not significant (ns), P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001; means in the same row with different superscripts are significantly different (P<0.05).

Table 3.5 – Partial sums of fatty acids (g/100 g fatty acids) and Δ9-indices of subcutaneous (Sub) and mesenteric (Mes) fats from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana				Barrosã					Significance Level						
	HS		LS		HS		LS									
	Sub	Mes	Sub	Mes	Sub	Mes	Sub	Mes	SEM	B	D	FD	B×D	B×FD	D×FD	B×D×FD
Partial sums																
Σ SFA	46.9^{a}	63.9^{b}	44.8^{a}	62.1^{bd}	39.6^{c}	61.3^{bd}	39.2^{c}	59.4^{d}	1.06	***	ns	***	ns	**	ns	ns
Σ MUFA	44.9^{a}	26.1^{b}	46.0^{a}	27.3^{b}	50.6^{c}	27.6^{bd}	51.5^{c}	30.3^{d}	1.06	***	ns	***	ns	**	ns	ns
Σ TFA	2.33^{a}	3.69^{b}	3.23^{bd}	4.32^{c}	2.95^{d}	4.51^{c}	2.98^{d}	4.52^{c}	0.219	ns	ns	***	ns	*	ns	ns
Σ BCFA	2.69^{a}	3.15^{b}	2.06^{c}	2.38^{d}	2.77^{a}	2.86^{a}	2.25^{cd}	2.24^{cd}	0.082	ns	***	***	ns	***	ns	ns
Σ PUFA	1.80^{a}	2.30^{ce}	2.58^{be}	3.09^{d}	2.07^{c}	2.43^{e}	2.11^{c}	2.49^{e}	0.109	ns	***	***	***	*	ns	ns
n-6 PUFA	1.53^{a}	1.97^{bef}	2.32^{cf}	2.82^{d}	1.75^{b}	2.06^{ef}	1.86^{be}	2.22^{f}	0.103	*	***	***	***	**	ns	ns
n-3 PUFA	0.273^{ac}	0.323^{b}	0.259^{ac}	0.274^{c}	0.324^{d}	0.379^{e}	0.248^{abc}	0.278^{ac}	0.012	*	***	***	**	ns	*	ns
n-6/*n*-3	5.58^{a}	6.14^{a}	9.07^{b}	10.3^{c}	5.42^{a}	5.49^{a}	7.51^{d}	8.09^{d}	0.337	***	***	***	**	ns	*	ns
Σ Unidentified	0.890	0.561	0.887	0.597	0.983	0.830	0.909	0.532	0.112	ns	ns	**	ns	ns	ns	ns
Desaturase indices																
Δ^9-index 14	16.8^{a}	4.05^{c}	18.7^{a}	4.20^{c}	24.1^{b}	4.46^{c}	26.1^{b}	4.87^{c}	1.29	***	ns	***	ns	***	ns	ns
Δ^9-index 16	12.9^{a}	3.69^{c}	12.5^{a}	3.79^{c}	17.2^{b}	3.83^{c}	17.7^{b}	4.17^{c}	0.690	***	ns	***	ns	***	ns	ns
Δ^9-index 18	69.6	39.2	72.9	41.3	76.5	43.2	79.3	46.6	1.43	***	*	***	ns	ns	ns	ns
Δ^9-index 18:1*t*11	22.5^{a}	0.723^{d}	20.7^{ab}	0.615^{d}	30.5^{bc}	1.35^{de}	32.4^{c}	1.37^{e}	0.994	***	ns	***	ns	***	ns	ns
Δ^9-index total	45.2^{a}	25.8^{b}	47.4^{a}	27.1^{bd}	52.5^{c}	27.9^{b}	53.1^{c}	30.1^{d}	1.15	***	ns	***	ns	**	ns	ns
Elongation ratio§	1.77^{a}	2.20^{b}	1.84^{ac}	2.25^{b}	1.99^{ac}	2.16^{b}	1.84^{ac}	2.17^{b}	0.069	ns	ns	***	ns	*	ns	ns

Σ SFA = sum of 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0; Σ MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 18:1*c*12, 18:1*c*13, 18:1*c*15, 19:1 and 20:1*c*11; Σ TFA = sum of 18:1*t*6-*t*8, 18:1*t*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*t*16+*c*14 and 18:2*t*11*c*15; Σ PUFA = sum of 18:2*n*-6, 18:3*n*-3, 20:3*n*-6 and 20:4*n*-6; Σ BCFA = sum of *i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0 and *i*18:0; Σ *n*-6 = sum of 18:2*n*-6, 20:2*n*-6 and 20:4*n*-6; Σ *n*-3 = 18:3*n*-3; Δ^9-index 14:0=(14:1c9×100)/(14:0+14:1c9); Δ^9-index 16:0=(16:1c9×100)/(16:0+16:1c9); Δ^9-index 18:0=(18:1c9×100)/(18:0+18:1c9); Δ^9-index 18:1t11=(18:2c9,t11×100)/(18:1t11+18:2c9,t11). Δ^9-index total = (14:1c9 + 16:1c9 + 18:1c9 + CLA *c*9,*t*11) ×100/(14:1c9 + 16:1c9 + 18:1c9 + CLA *c*9,*t*11+ 14:0 + 16:0 + 18:0 + 18:1 *t*11); § Elongation ratio = (18:0+18:1*t*6-*t*8+18:1*t*9+18:1*t*10+18:1*t*11+18:1*t*12+18:1*c*9+18:1*c*11+18:1*c*12+18:1*c*13+18:1*t*16+*c*14+18:1*c*15)//(16:0+16:1*c*7+16:1*c*9); D = diet; B = breed; FD = fat depot. Significance level: not significant (ns), *P*>0.05; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; means in the same row with different superscripts are significantly different (*P*<0.05).

Table 3.6 – Loadings for the first four principal components (PC)

Variables	PC1	PC2	PC3	PC4
12:0	0.536	0.240	-0.171	0.557
14:0	0.128	0.167	-0.662	0.561
*i*15:0	0.520	0.781	0.041	0.072
*a*15:0	0.839	0.349	0.025	0.057
14:1*c*9	-0.888	0.106	-0.076	0.293
15:0	0.769	0.188	-0.162	0.432
*i*16:0	0.597	0.698	0.030	0.012
16:0	0.291	0.076	-0.737	0.182
i17:0	0.701	0.306	0.430	0.101
16:1*c*7	0.697	0.159	0.263	0.474
16:1*c9*	-0.922	0.157	-0.052	0.223
*a*17:0	-0.685	0.456	0.137	0.202
17:0	0.842	-0.216	-0.128	-0.082
*i*18:0	0.329	0.601	-0.024	-0.337
17:1*c*9	-0.920	0.011	-0.102	0.187
18:0	0.944	-0.082	0.068	-0.218
18:1*t*6-*t*8	0.599	-0.632	0.212	0.197
18:1*t*9	0.278	-0.479	0.585	0.177
18:1*t*10	-0.036	-0.820	-0.146	0.221
18:1*t*11	0.683	-0.151	0.426	0.162
18:1*t*12	0.705	-0.279	0.253	0.111
18:1*c*9	-0.957	0.065	0.083	-0.002
18:1*c*11	-0.858	0.091	0.078	0.127
18:1*c*12	-0.732	-0.078	0.328	0.162
18:1*c*13	-0.913	-0.033	0.080	0.126
18:1*t*16+*c*14	0.775	0.212	0.365	0.148
18:1*c*15	-0.079	-0.407	-0.050	0.496
18:2*n*-6	0.370	-0.671	0.075	0.222
19:1	-0.735	0.150	0.283	0.327
20:0	0.908	-0.030	0.203	-0.020
18:3*n*-3	0.463	0.479	0.252	0.317
20:1*c*11	-0.719	-0.067	0.290	-0.085
18:2*c*9*t*11	-0.779	0.210	0.298	0.261
20:4*n*-6	0.189	-0.127	0.169	-0.094
Adipocytes area	-0.475	0.218	0.423	-0.031
Adipocytes number	0.480	-0.188	-0.412	0.009
Proportion of variance (%)	45.3	12.8	8.36	6.37
Cumulative variance (%)	45.3	58.1	66.5	72.8

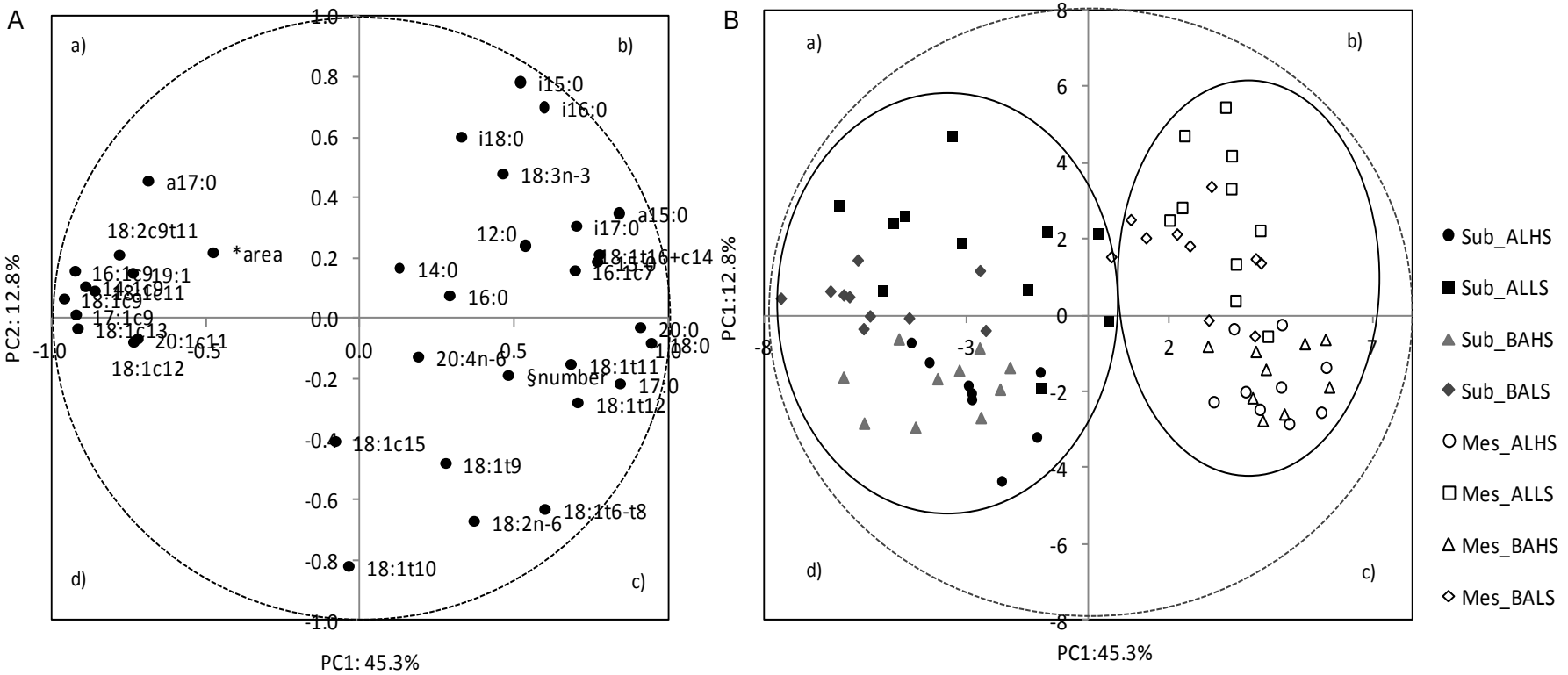

Figure 3.*2* – Loading plot of the first and second principal components (PC) of the pooled data (A) and component's score vectors (B) for subcutaneous (Sub) and mesenteric (Mes) fats from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low (LS) silage diets
*Adipocytes area; § Adipocytes number

exception was the Δ^9-18 index, which was influenced by breed (P<0.001), fat depot (P<0.001) and diet (P<0.05). This former index was higher in subcutaneous fat of low silage fed Barrosã bulls. In contrast to the desaturation indices, neither breed nor diet influenced the elongation ratio, which showed to be strongly influenced by the fat depot (P<0.001), as well as its interaction with breed (P<0.05).

3.3.4 MUFA discriminate the subcutaneous fat from the mesenteric fat

A PCA was applied to a data set of cellularity and fatty acid composition parameters in order to describe the variability of the pooled data into two dimensions (Fig. 3.2A). The score plot of the first two components explains 58.1% of the total variability, with 45.3% for PC1 and 12.8% for PC2 (Table 3.6). The score plot showed a clear separation of adipocytes number from MUFA, which in turn were closely associated with adipocytes area. The cell number was located near the arachidonic acid and some of the 18:2*n*-6 ruminal biohydrogenation related fatty acids (18:1*t*11 and 18:*t*12). Most of the MUFA were allocated on the left side of the plot (quadrants *a* and *d*), clearly separated from the other variables, showing negative scores for the PC1 and little influence on the PC2.

The PC2 clearly distinguished BCFA, located in the upper part of the plot, from adipocytes number, located in the lower part of the graphic (quadrant *c*). In quadrant *b*, a cluster was defined by *a*15:1, *i*16:0, 16:1*c*7, 18:1*t*16+*c*14 and *i*17:0 fatty acids.

The score plot depicted in Figure 3.2B shows the location of subcutaneous and mesenteric adipose fats in the multivariate space of the first two PCs. These scores were notably arranged in two clusters, corresponding to both fat depots. In contrast, no clear discrimination between breeds, Alentejana and Barrosã, or diets, 30/70% and 70/30% of silage and concentrate, was achieved.

3.4 Discussion

The economical and physiological importance of fat deposition in meat animal production has long been recognized (Robelin, 1981; Hood, 1982). Subcutaneous fat, along with the intermuscular fat, is the largest adipose tissue depot (Cianzio *et al.*, 1985) with the highest lipogenic activity (Poulos *et al.*, 2010), whereas mesenteric fat displays distinctive immune-response potential (Virtue & Vidal-Puig, 2008; Mukesh *et al.*, 2010). Nonetheless, information concerning the biology and regulation of each fat depot is limited.

In the present study, live slaughter weight was determined by breed, as Alentejana and Barrosã bulls are quite distinct in morphological characteristics (Silva *et al.*, 1998). As for the

diet, it showed no impact on live slaughter weight. Regarding the deposition of adipose tissue, there was no influence from breed or diet on the subcutaneous fat. The analysis of the visceral fat partitioning revealed diet as the major influencing factor, in particular for mesenteric fat in low silage fed animals. The critical factor affecting glucose levels in plasma was the breed, with higher values in Alentejana than in Barrosã bulls. It is well known that ruminants have typical insulin resistance compared to monogastric animals. Insulin concentrations were affected by diet, as low forage diets fed to both breeds promoted higher values of this hormone. In ruminants, dietary carbohydrates are fermented into volatile fatty acids by ruminal microorganisms, and the propionate formed is used as a primary precursor for gluconeogenesis (Wan *et al.*, 2009). Therefore, propionate from rumen fermentation is largely associated with body fat deposition, as it promotes lipogenesis through the secretion of insulin.

Leptin is synthesized and released into the bloodstream in direct proportion to the amount of body fat, reflecting primarily the TAG content of lipid depots, but also functioning as a sensor of energy balance (Houseknecht *et al.*, 1998; Chilliard *et al.*, 2005). The systemic leptin levels are strongly associated with mRNA levels in subcutaneous adipose tissue and cellularity (Delavaud *et al.*, 2002; Higashiyama *et al.*, 2003). Herein, plasma leptin levels were unchanged across dietary groups, which is consistent with the lack of variation in subcutaneous fat tissue parameters.

IL-6, a primary pro-inflammatory interleukin, has been reported to be increased in fat animals (Wang *et al.*, 2008). In this study, plasma concentration of IL-6 was influenced by breed, with higher levels in Barrosã than in Alentejana bulls. Even if this might reflect the breed effect observed in one of the largest adipocyte classes (from 144s00 to 16200 μm^2), it should be underlined that IL-6 is not exclusively adipose tissue-derived. In fact, only 10% of this cytokine is produced by adipocytes (Ronti *et al.*, 2006).

The histological characterization of fat depots showed no significant differences between breeds. Yet, fat depot was of extreme importance regarding cellularity and fatty acid profile. The increase of adipocytes area in the subcutaneous fat relatively to the mesenteric fat might be an indicator of an early adipocytes differentiation in this fat depot. Apart from this, no other significant effects were observed on fat depot cellularity. Concomitantly, there were also no significant differences across the experimental groups regarding plasma TAG.

The amount of adipose tissue in animals during growth is related both with hyperplasia and hypertrophy, although growth of different adipose tissues in cattle after birth is more attributable to adipocytes hypertrophy (Alzón *et al.*, 2007). According to Robelin (1981), subcutaneous adipose tissues have the highest relative growth and appear to be the youngest on a cellularity basis: small-sized cells, high and late hyperplasia, in comparison to internal fat depots. In the same experiment, kidney and peritoneal adipose tissues were used

as representative of the internal fat depots (Robelin, 1981). Similar results have been reported by Mendizabal *et al.* (1999) in a study concerning adipocytes size of subcutaneous and two visceral fat depots (omental and perirenal) from several bovine breeds. These authors observed that subcutaneous fat had smaller adipocytes than both omental and perirenal fat depots. Nevertheless, both studies failed to characterize the mesenteric fat depot. In contrast, our results showed that the subcutaneous fat had larger adipocytes than the chosen visceral fat depot, in accordance to Pike and Roberts (1984) findings. Thus, the differential cellularity of mesenteric fat from other visceral fat depots could result from distinct lipogenic activities.

The relationship between adipocytes size and fat depot mass has been used to establish which process, hypertrophy, hyperplasia or both, is responsible for fat depot development (Mendizabal *et al.*, 1999). A high correlation coefficient indicates the prevalence of hypertrophy, whereas a low correlation coefficient reveals the occurrence of both processes. The lack of a significant correlation points to hyperplasia as the main contributor to tissue development. In this study, the correlation coefficients between adipocytes area and fat depot mass might suggest that while the development of mesenteric fat may be due to both hypertrophy and hyperplasia, the subcutaneous fat development may be mostly attributed to hyperplasia.

The highest amount of fatty acids deposition was observed in the mesenteric fat of Alentejana breed. This might be due to differences in fatty acid deposition mechanisms between breeds, with Alentejana accumulating higher amounts of fatty acids within this internal fat depot. When comparing both adipose tissues, the mesenteric fat depot was more saturated and richer in TFA, PUFA and BCFA, whereas subcutaneous fat depot contained more MUFA. Diet was the major factor affecting PUFA and BCFA contents, while SFA and MUFA were breed-related. The high MUFA content in the subcutaneous adipose tissue was already reported (Aldai *et al.*, 2007) as a consequence of elevated stearoyl-coA Δ9-desaturase activity. The ruminal transformation of dietary lipids plays a key role in determining the fatty acid composition of ruminant products. Diets containing high proportions of non-structural carbohydrates, as starch, but low amount of fibre promote usually less extensive biohydrogenation (Palmquist *et al.*, 2005). In fact, BCFA percentages in both fat depots were higher in high silage than in low silage fed animals. According to Aldai *et al.* (2007), BCFA are higher in leaner animals and, in fact, no effect of breed was observed. This is concomitant with other parameters measured in this study, namely fat depots mass, cellularity or leptin levels. On the other hand, dietary starch is negatively correlated with *i*14:0, *i*15:0 and *i*16:0 fatty acids (Bessa *et al.*, 2007). Indeed, the higher proportions of starch observed in low silage diet promoted a decrease in the aforementioned BCFA relatively to high silage diet.

Typically, feeding maize silages results in a high *n*-6/*n*-3 ratio in body fat due to its high content in 18:2*n*-6 (O'Sullivan *et al.*, 2002). It also increases *de novo* synthesis of SFA from starch. In ruminants, the ratio of 18:0/18:2*n*-6 in the adipose tissue declines, as fattening proceeds (Wood *et al.*, 2008). Indeed, high silage fed Barrosã bulls showed the lowest value of the above mentioned ratio (5.42) in the subcutaneous fat, which reinforces the results regarding total fatty acids. Breed was also an important factor influencing CLA deposition and, in both fat depots, those changes occurred in parallel with vaccenic acid (18:1*t*11) variations. Shen *et al.* (Shen *et al.*, 2007) and Dance *et al.* (2009) reported breed-specific responses regarding vaccenic acid and CLA contents. Hishikawa *et al.* (2005) and Gotoh *et al.* (2009) suggested a distinct regulation of adipose tissue development promoted by differential expression of fat-related genes in subcutaneous and visceral adipose tissues. The *c*9,*t*11 CLA isomer content in adipose tissue derives from local biosynthesis from vaccenic acid by stearoyl-coA Δ-9 desaturase enzyme. Significant differences between breeds and fat depots were observed in the vaccenic acid, which were consequently reflected on the *c*9,*t*11 CLA isomer proportions. In a study by Garcia *et al.* (2003), the proportion of CLA in the adipose tissue increased with fatness, as did vaccenic acid.

Lipids of forages and feedstuffs, mainly rich in 18:3*n*-3 and 18:2*n*-6, once in the rumen, are readily hydrolysed to NEFA by microbial lipases. After hydrolysis, the released non-esterified PUFA undergo a series of enzymatic steps leading to the formation of more SFA, until 18:0 fatty acid. During biohydrogenation, multiple intermediates of fatty acids are formed, such as CLA isomers and 18:1*t*11. The *c*9,*t*11 CLA isomer was the most abundant across breeds, diets and fat depots, followed by the *t*7,*c*9, as reported by Fritsche *et al.* (2000) for beef and milk. Breed showed a clear influence on CLA isomers' proportions, affecting eleven of the fifteen isomers. Dannenberger *et al.* (2005) also reported significant differences between breeds on CLA isomers distribution in distinct fat depots. The influence of diet was also noticeable on the main CLA isomers. The low silage diet promoted lower proportions of *t*11,*t*13 and *t*11,*c*13 CLA isomers compared to the high silage diet. Similar results were described when pasture and concentrate diets were compared (Dannenberger *et al.*, 2005). High silage feeding decreased the proportions of *t*10,*c*12 CLA isomer in comparison to low silage, which is in accordance with Dannenberger *et al.* (2005). Overall, the subcutaneous fat showed higher deposition of CLA isomers. This is consistent with reports on the highest lipogenic activity in this adipose tissue depot (Poulos *et al.*, 2010). Nevertheless, Eguinoa and colleagues (2003), in a study concerning the subcutaneous, intermuscular, omental and perirenal fat depots, reported that the visceral depots exhibited higher lipogenic enzyme activities per cell than the subcutaneous fat. However, when the catalytic activity per cell was adjusted for cell size, the subcutaneous depot had greater enzyme activities than omental and perirenal fats.

The higher and lower proportions of SFA and MUFA, respectively, in mesenteric fat, relatively to subcutaneous fat, suggest a higher lipogenic activity in the former. This explanation is in agreement with higher Δ^9-desaturase indices, key indicators of lipogenic activity (Siebert *et al.*, 2003), found in the subcutaneous fat, in comparison to the mesenteric fat. The breed-related variations in the Δ^9-desaturase indices observed in this study indicate a differential Δ^9-desaturase activity, favouring Barrosã breed. This might be a consequence of a higher lipogenic activity in Barrosã, an early maturing breed, in comparison to Alentejana, known to be late maturing. In addition, fat depot and its interaction with breed, showed an influence on the elongation index. This suggests differential Δ^5 and Δ^6 desaturase activities between the subcutaneous and mesenteric fat depots. These enzymes are involved in the conversion of C18 PUFA to their long-chain derivatives.

The PCA established the relationships between cellularity and fatty acid composition of both adipose tissues, along with associations among fatty acids. This statistical approach confirmed the subcutaneous and mesenteric adipose tissues contrasting features, which arose from the analysis of variance. MUFA, closely associated with adipocytes area, was the major contributor for the distinction between fat depots which reinforces the concept of a differential metabolic and desaturase activity. Considering that Δ^9 is the key enzyme converting SFA into MUFA, and bearing in mind the close association between most MUFA and adipocytes area, it would be plausible to speculate that higher adipocytes area found in the subcutaneous fat is responsible for a higher desaturation activity.

The genetic background strongly influences lipid incorporation, as shown through the fatty acid composition and CLA isomeric profile. However, contrarily to expected, the influence of breed on subcutaneous and mesenteric fat deposition and partitioning was not significant. Feeding different silage/concentrate ratios impacted on PUFA, BCFA and CLA isomers, which were the classes of fatty acids most sensitive to diet composition. Taking into account all results herein presented, the major factor determining lipid deposition is fat depot. Moreover, the distinct cellularity observed in subcutaneous and mesenteric fats from bulls might reflect a differential dynamics between hypertrophy and hyperplasia processes in these two adipose tissue depots.

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CHAPTER 4

Gene regulation of fatty acid composition in muscle and subcutaneous adipose tissues

Expression of genes controlling fat deposition in two genetically diverse beef cattle breeds fed high or low silage diets

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ASH Costa collaborated in the tissue sampling and laboratory work. ASH Costa was responsible for the statistical analysis. ASH Costa also participated in the interpretation of the results and preparation of the manuscript.

Abstract

Both genetic background and finishing system can alter fat deposition, thus indicating their influence on adipogenic and lipogenic factors. However, the molecular mechanisms underlying fat deposition and fatty acid composition in beef cattle are not fully understood. This study aimed to assess the effect of breed and dietary silage level on the expression patterns of key genes controlling lipid metabolism in subcutaneous adipose tissue (SAT) and LL muscle of cattle. To that purpose, forty bulls from two genetically diverse Portuguese bovine breeds with distinct maturity rates, Alentejana and Barrosã, were selected and fed either low (30% maize silage/70% concentrate) or high silage (70% maize silage/30% concentrate) diets.The results suggested that enhanced deposition of fatty acids in the SAT from Barrosã bulls, when compared to Alentejana, could be due to higher expression levels of lipogenesis (*SCD* and *LPL*) and β-oxidation (*CRAT*) related genes. Our results also indicated that *SREBF1* expression in the SAT is increased by feeding the low silage diet. Together, these results point out to a higher lipid turnover in the SAT of Barrosã bulls when compared to Alentejana. In turn, lipid deposition in the LL muscle is related to the expression of adipogenic (*PPARG* and *FABP4*) and lipogenic (*ACACA* and *SCD*) genes. The positive correlation between *ACACA* expression levels and total lipids, as well TFA, points to ACACA as a major player in intramuscular deposition in ruminants. Moreover, results reinforce the role of *FABP4* in intramuscular fat development and the SAT as the major site for lipid

metabolism in ruminants. Overall, the results showed that SAT and LL muscle fatty acid composition are mostly dependent on the genetic background. In addition, dietary silage level impacted on muscle lipid metabolism to a greater extent than on that of SAT, as evaluated by gene expression levels of adipogenic and lipogenic factors. Moreover, the response to diet composition evaluated through mRNA levels and fatty acid composition showed interesting differences between Alentejana and Barrosã bulls. These findings provide evidence that the genetic background should be taken into account while devising diet-based strategies to manipulate fatty acid composition of beef cattle tissues.

4.1 Background

During the last decades consumers have started demanding animal products of low fat and high polyunsaturated fatty acids content, while maintaining high and consistent quality (Hocquette *et al.*, 2011; Scollan *et al.*, 2006). To that purpose, research has been conducted on ruminant's adipogenesis and lipogenesis in order to improve both the production efficiency and beef quality. Adipose tissue is involved in the regulation of body homeostasis, particularly in energy metabolism, storage and expenditure. In cattle, fatty acid type and amount in muscles are directly associated with meat quality and its value. In fact, subcutaneous and intramuscular adipose tissues are the most important fat depots concerning meat quality traits. It is desirable that cattle carcasses have minimal amounts of fat stored in SAT, without a detrimental decrease in intramuscular fat (Pickworth *et al.*, 2011). This can be achieved only if the regulation of lipid deposition in intramuscular and other fat depots differs substantially. Despite being the main site for *de novo* fatty acid and TAG synthesis in ruminants (Hiller *et al.*, 2012), the SAT is also the most energetically inefficient fat depot and, therefore, considered an economic loss. However, while during the last decade knowledge of rodents and human fat physiology has progressed rapidly (Roh *et al.*, 2006), the same information regarding ruminant species is very limited.

The expression level of adipogenic and lipogenic genes in adipose tissues is regulated by a number of transcription factors (Mannen*,* 2011), whose differential expression is known to play a key role in lipid metabolism of cattle adipocytes (Taniguchi *et al.*, 2008). Adipogenesis, lipogenesis, and lipolysis occur through the interaction of endogenous genetic mechanisms (mediated through gene expression and regulated by intrinsic factors), external controls (endocrine agents, extrinsic factors and nutritional metabolites), as well as local interactions within cells in a fat depot (Fernyhough *et al.*, 2005). Despite the intricacies of lipogenesis and lipolysis, the role of some genes has been elucidated and confirmed to be related to fatty acid composition in cattle (Mannen, 2011). Potential regulatory mechanisms involved in the

fatty acid deposition are lipogenic (*ACACA*, *LPL*, *FABP4* and *SCD*) and oxidative (*CPT1B* and *CRAT*) genes, as well as transcription regulators (*PPARA*, *PPARG* and *SREBF1*). These genes have been described (Wang *et al.*, 2005; Zhang *et al.*, 2010; Lee *et al.*, 2010) for their roles and expression patterns during adipocyte differentiation, namely in studies comparing the regulation of adipose tissue deposition in distinct cattle breeds (Wang *et al.*, 2005; Lehnert *et al.*, 2007).

Genetic factors underlying both the deposition and the turnover of individual fatty acids are not fully understood, although breed has been found to influence beef fatty acid composition (Aldai *et al.*, 2007). Fatty acid composition in meat-producing animals is recognised to have implications on the nutritional and organoleptic properties of meat, as well as in its technological quality (Wood *et al*., 2008). Ruminant products can be an additional source of the beneficial long-chain *n*-3 PUFA (EPA and DHA) for human diets (Scollan *et al.*, 2006) when the consumption of *n*-3 PUFA-rich foods, such as fish, is low. In addition, ruminant meats are major dietary sources of docosapentaenoic acid (22:5*n*-3). Thus, genetic selection and breeding of animals with a desirable meat fatty composition may provide a source of beneficial fatty acids for human consumption (Widman *et al.*, 2011). Comparative differences of beef cattle present a unique resource to study several aspects of lipid metabolism. In addition to genetic factors, finishing systems can dramatically alter fat deposition (Pavan *et al.*, 2008), thus indicating that lipogenic activity is influenced by the dietary energy level, the energy source and, possibly, the forage to concentrate ratio. However, fatty acid composition in ruminant animals, unlike in monogastrics, is much less dependent on the diet, as a consequence of dietary fatty acid metabolism (*i.e.*, isomerisation, biohydrogenation) within the rumen (Hoashi *et al.*, 2008).

The biochemical processes and the molecular background affecting genetic variability of the complex trait of fat content and fatty acid composition are not yet fully understood, particularly with regard to European cattle breeds, because most of the recent studies have been performed on the specific genetic background of Japanese Black cattle (Widman *et al.*, 2011). Therefore, we conducted a trial with bulls from two Portuguese genetically diverse breeds (Beja-Pereira *et al.*, 2003) with distinct maturity rates, Alentejana and Barrosã, fed high or low silage diets. The working hypothesis of the present paper was that the expression of key genes controlling lipid metabolism during the finishing phase of cattle is breed-specific (Alentejana *vs*. Barrosã) and diet-modulated (high *vs*. low silage). Furthermore, given the distinct roles of subcutaneous and intramuscular fat depots in cattle lipid metabolism, the tissue-specific variations were also investigated through the analysis of both SAT and muscle gene expression patterns. Finally, the breed-, diet- and tissue-specific relationships among the expression level of these genes and fat content and composition were also assessed.

4.2 Material and Methods

4.2.1 Animals and experimental diets

Details concerning the experimental design, animals and the composition of experimental diets are provided in Chapter 2, section 2.2. Results concerning productive performance were also described previously in Chapter 2. Briefly, Alentejana bulls fed the high silage diet had an average weight of 622±17.7 kg at slaughter, whereas the average weight for those fed the low silage diet was 636±29.7 kg. Regarding Barrosã bulls, the weight at slaughter was 457±8.88 kg for those fed the high silage diet and 497±23.0 kg for bulls fed the low silage diet. DM intake and average daily gain were higher in Alentejana when compared to Barrosã bulls, although feed efficiency was similar across experimental groups. Fatness scores were higher in Barrosã than in Alentejana bulls. Moreover, the low silage fed animals had higher fatness scores than those fed the high silage diet.

4.2.2 Sample collection

Immediately after slaughter, samples of LL muscle and SAT for gene expression analysis were collected from the right side of carcass at the 5th lumbar vertebra level, rinsed with sterile RNAse-free cold water solution, cut into small pieces (thickness of ~0.3 cm), stabilised in RNA Later solution (Qiagen, Hilden, Germany) and subsequently stored at –80 ºC. A second sample of SAT was vacuum-packed and stored at –20 ºC, until lipid extraction and determination of fatty acid composition.

Carcasses were suspended from the Achilles tendon, chilled at 10 ºC for 24 hours and aged during 8 days at 2 °C. The left half carcass was subsequently separated into commercial joints. The LL muscle samples (*ca*. 200 g) were collected, trimmed of connective and adipose tissues before being blended in a food processor, vacuum packed and stored at –20 ºC until lipid analysis.

4.2.3 Total lipid content and fatty acid composition

SAT and LL muscle samples were lyophilised (–60 °C and 2.0 hPa), maintained at -20 °C (SAT) or dissecated at room temperature (muscle) and analysed within two weeks. Total lipids were extracted from LL and SAT as described in chapters 2 and 3, respectively.

Fatty acids were then converted to methyl esters and the resulting FAME were then analysed by GC as described in chapter 3, section 3.2.

4.2.4 Total RNA extraction

Frozen tissue samples were homogenized with an Ultra-Turrax® homogenizer (IKA-Labortechnik, Staufen, Germany). For SAT samples, total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen Inc, Hilden, Germany) according to the manufacturer's protocol. Total RNA was extracted from muscle samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the RNeasy Mini Kit (Qiagen Inc), according to the manufacturer's protocol. To exclude possible amplification of contaminating genomic DNA, an additional step of DNase digestion was performed with the RNase-free DNase Set (Qiagen Inc.), incubating samples with DNase for 15 min at room temperature. Total RNA extracts were immediately analyzed for quantity (OD260nm) and purity (OD260nm/OD280nm) (NanoDrop ND-2000c, Peqlab GmbH, Erlangen, Germany). RNA aliquots were stored at –80 ºC and until further analysis.

4.2.5 Synthesis of complementary DNA

Single-stranded cDNA was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Each 20 µl RT reation contained 250 ng (SAT) or 600 ng (muscle) of RNA template, 50 nM random RT Primer, 1×RT buffer, 0.25 mM of each dNTPs, 3.33 U/µl multiscribe reverse transcriptase and 0.25 U/µl RNase inhibitor, at temperatures of 25 ºC for 10 min, 37 ºC for 120 min, and 85 ºC for 5 min cDNA aliquots were stored at –20 °C.

4.2.6 Primer design and housekeeping gene stability evaluation

Primer Express software (Applied Biosystems, Foster City, CA, USA) was used to design primers fixing the amplicon length to 65–150 bp (Table 4.1). When possible, primers were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN at the National Center of Biotechnology Information. Prior to RT-qPCR the various sets of gene-specific primers were tested using a conventional PCR and run in a 2.5% agarose gel stained with ethidium bromide. Only those primers that presented a single band at the expected size in the gel, and thus no primer-dimer formation, were used for RT-qPCR. The accuracy of primer pairs was also evaluated by the presence of a unique

peak during the dissociation step at the end of RT-qPCR. A set of five candidate housekeeping genes was evaluated using geNorm and NormFinder, following the procedures described by Vandesompele *et al.* (2002) and Andersen *et al.* (2004), respectively. Therefore, target gene expression levels were normalised against the expression levels of the housekeeping genes, ribosomal protein large P0 (*RPLP0)* and peptidylprolyl isomerase B (*PPIB)* for SAT and muscle, respectively.

4.2.7 Real time quantitative polymerase chain reaction

The RT-qPCR was performed with the StepOne Plus™ Real-Time PCR System, using the Power SYBR® Green master mix (both Applied Biosystems, Foster City, CA, USA). Reaction mixes of 6.25 μL Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 1 μL of forward and reverse primers (160 nM) and 1 μL of diluted cDNA (1:10) template were pipetted into MicroAmp™ optical 96-Well reaction plates and sealed with optical caps (Applied Biosystems, Foster City, CA, USA). After an initial denaturation at 95 ºC for 10 min, a thermocycling program of 15 s at 95 ºC, 60 s at 60 ºC and 15 s at 95 ºC was applied (40 cycles). Total fluorescence data and dynamic well factors were continuously collected to generate background-subtracted amplification curves (StepOne™ Software v2.2.2, Applied Biosystems, Foster City, CA, USA). PCR analysis of cDNA samples was performed in duplicate, using no-transcription and no-template samples as controls. The specificity of the PCR amplification was confirmed by melting curve analysis and agarose gel electrophoresis of PCR products.

4.2.8 Data processing

The PCR efficiency was calculated for each primer set using the StepOnePlus PCR System software (Applied Biosystems), by amplifying 5-fold serial dilutions of pooled cDNA and run in triplicate. The efficiency curves were used to assess accuracy, linearity and efficiency of the PCR reaction. Efficiency (E) was calculated as E [%] = (10-1/slope of standard curve - 1) × 100. All primer sets exhibited an efficiency ranged between 85 and 110% and correlation coefficients were higher than 0.990.

The relative expression (RE) levels were calculated as a variation of the Livak method (2001), corrected for variation in amplification efficiency ($E = 10^{-1/slope}$), as shown bellow.

$$RE = E_{endogenous}^{(CT,endogenous)} / E_{target}^{(CT,target)}$$

Table 4.1 – Primer pairs sequences for quantitative real-time PCR[1-4]

Gene symbol	Full gene name	Acc. Number [1]	Primer pairs (5'-3')	Exons spanned	Product size (bp)
ACACA	acetyl-CoA carboxylase alpha	NM_174224	F: ttc acg tgg cct ggg tag a R: ttg tac ctg gat tct cct tca tct t	40-41	142
CPT1B	carnitine palmitoyltransferase 1B	NM_001034349	F: gcg act cca gtg gga cat tc R: aaa ggc agg aac tgg aag ca	12-13	114
CRAT	carnitine O-acetyltransferase	NM_001075587	F: ggc cca ccg agc cta cac R: atg gca atg gcg tag gag gt	12-13	138
FABP4	fatty acid-binding protein 4	NM_174314	F: tgg atg ata aga tgg tgc tgg a R: atg gag ttc gat gca aac gtc	3-4	114
LPL	lipoprotein lipase	NM_001075120	F: act gtg gct gag agc gag aac R: tct cca ata tcc acc tcc gtg ta	7-8	98
PPARA	peroxisome proliferator-activated receptor alpha	NM_001034036.1	F: agt gcc ttt cag ttg gga tgt c R: cgc ggt ttc gga atc ttc ta	2-3	125
PPARG	peroxisome proliferator-activated receptor gamma	NM_181024	F: tgt ctc ata atg cca tca ggt ttg R: tct ccg cta aca gct tct cct t	4-5	66
SCD	stearoyl-CoA desaturase	NM_173959	F: cca tca acc ccc gag aga at R: aag gtg tgg tgg tag ttg tgg aa	5-6	76
SREBF1	sterol regulatory element binding transcription factor 1	NM_001113302	F: agc ctg gca atg tgt gag aag R: caa gga gca ggt cac aca gga	13-14	115
PPIB[2]	peptidylprolyl isomerase B	NM_174152	F: tcc gtc ttc ttc ctg ctg ttg R: cca att cgc agg tca aag tac	1-2	98
RPLP0[3]	ribosomal protein P0	NM_001012682	F: gca ttc ccg ctt cct gg R: gcg ctt gta ccc att gat ga	5-6	109

[1] Entrez Gene, National Center for Biotechnology Information (NCBI)
[2] housekeeping gene for muscle
[3] housekeeping gene for subcutaneous adipose tissue
[4] F: forward primer and R: reverse primer.

4.2.9 Statistical analysis

Statistical analyses were carried out with the Statistical Analysis Systems software package, version 9.2, (SAS Institute, Cary, NC, USA). All statistical analyses were performed based on a 2 × 2 factorial arrangement of breed (Alentejana and Barrosã purebreds), diet (high and low silage diets) and their respective interaction. The variances were tested for heteroscedasticity and, for most parameters, variance was found to be heterogeneous. Therefore, subsequent data analysis was performed in order to account for heterogeneous variance. The general Satterthwaite approximation was computed in a mixed-effects regression model (PROC MIXED), with breed, diet and their interaction as fixed effects.

The slaughter weight, total lipids content in the muscle and SAT were tested as covariates, but only total lipids was retained. Whenever the use of a covariate was necessary, the structure of the covariate model was determined according to the procedures described by Milliken and Johnson (2002), ranging from a simple slope model to individual slopes for each diet×breed combinations. Given that significant differences in covariate ranges were intrinsically associated to each breed, each variable was adjusted and compared with the mean covariate value of each breed (Milliken & Johnson, 2002). When significant effects were detected, least square means were determined using the LSMEANS option, with no correction for multiple comparisons. Differences were declared significant at $P<0.05$ and tendencies discussed at $P<0.10$.

Pearson correlation coefficients were calculated using the CORR procedure of SAS. Whenever necessary, adjusted variables to the IMF or the amount of subcutaneous adipose tissue in the leg joint were used to compute Pearson correlations, for the muscle and SAT samples respectively.

The relationships between cellularity and fatty acid composition in both depots were assessed by the PCA, using the PRINCOMP procedure of SAS. The PCA was applied to a data set of 78 samples and 17 variables to reduce the dimensionality of the data set and to describe the variability of data. The PCA was used to examine the relationship between the fatty acid composition and relative gene expression levels, enabling not only plots of the relationship between the variables but also attempting to explain those relationships. The analysis was based on the correlation matrix and the principal components which explained at least 5% for the total variance and had eigenvalues were greater than one were retained.

4.3 Results

4.3.1 Fatty acid composition of subcutaneous adipose tissue

Total lipid content and percentages of main fatty acids (*i.e.*, those >1% of total fatty acids and the main CLA isomer, 18:2*c*9*t*11, in the SAT from the four experimental groups are presented in Table 4.2. The content of total lipids was higher (P=0.002) in the SAT of Barrosã breed when compared to Alentejana bulls. Barrosã bulls had lower 14:0 (P=0.024), 16:0 (P<0.001) and 18:0 (P<0.001) fatty acid proportions in the SAT than the Alentejana bulls. Breed also determined the proportions of 16:1*c*9, *a*17:0, 18:1*t*11, 18:1*c*9 and 18:2*c*9*t*11 fatty acids in the SAT, with the Barrosã breed having higher values when compared to the Alentejana bulls (P<0.01). TFA were higher in Barrosã than in Alentejana bulls (P=0.004). Diet influenced the proportions of *a*17:0 and 18:0 (P<0.001 and P=0.034, respectively), with the high silage fed bulls presenting the highest values. Animals fed the low silage diet had the highest proportions of TFA (P=0.037). The branched chain fatty acids (BCFA), which are closely related to the rumen activity, were higher in animals fed the high silage diets (P<0.001). A breed×diet interaction was found for the fatty acid 18:2*n*-6 (P=0.002), with the Alentejana bulls fed the low silage diet presenting the highest proportion. While Alentejana bulls had higher proportions of SFA but lower monounsaturated fatty acids (MUFA) percentages, the inverse pattern was observed in the Barrosã bulls (P<0.001). Both total PUFA and *n*-6 PUFA percentages were higher in the Alentejana bulls fed the low silage diet, whereas in the SAT from Barrosã bulls no variation between diets was observed (breed×diet, P=0.001). The percentages of *n*-3 PUFA were the highest in the SAT from Barrosã bulls fed the high silage diet (P=0.012).

4.3.2 Fatty acid composition of muscle

The values for total lipids and main fatty acids (*i.e.*, those >1% of total fatty acids and 18:2*c*9*t*11) in the muscle from the four experimental groups are presented in Table 4.3. Total lipid contents were higher in the muscle from Barrosã bulls fed the low silage diet in comparison to those fed the high silage diet, whereas no variation was observed for the Alentejana bulls (breed×diet, P=0.001). The 14:0 proportion in the muscle was higher (P<0.001) in the Barrosã breed when compared to the Alentejana bulls. Breed also influenced the percentages of 16:1*c*9, 18:1*c*9 and 18:2*c*9*t*11 in the muscle, with the Barrosã having higher values when compared to the Alentejana breed (P<0.001). Barrosã bulls fed

the low silage diet had (P=0.037) the highest proportions of SFA. Both MUFA and TFA proportions were higher (P<0.001) in Barrosã when compared to Alentejana bulls.
Alentejana bulls had the highest percentages of total, *n*-3 and *n*-6 PUFA (P<0.001). The 14:0 percentages were higher (P=0.003) in the low silage than in the high silage fed bulls. Barrosã bulls fed the low silage diet had the highest 16:0 fatty acid proportions (breed×diet, P=0.003). Deposition of 20:4*n*-6 varied according to breed and diet (P<0.001 and P=0.001, respectively), with Alentejana having higher percentages than Barrosã bulls, and low silage promoting lower deposition in comparison to high silage diet. Feeding the low silage diet resulted in higher percentages (P=0.038) of TFA in comparison to the high silage diet. The BCFA were higher (P<0.001) in animals fed on the high silage diet. Bulls fed the high silage diet had higher *n*-3 PUFA percentages (P<0.001) than those fed the low silage diet. The Barrosã bulls fed the low silage diet had the lowest percentages of 18:2*n*-6 (breed×diet, P=0.007). The deposition of 18:0 was similar (P>0.05) across the four experimental groups.

4.3.3 Gene expression in subcutaneous adipose tissue

The relative mRNA expression levels of the nine lipid metabolism key factors analysed in the SAT are presented in Figure 4.1. *CRAT*, *LPL* and *SCD* showed higher expression levels in the Barrosã breed when compared to the Alentejana animals (P<0.05), corresponding to a fold-change of 1.46, 1.56 and 1.74, respectively. In addition, the Barrosã breed tended (P=0.081) to have higher expression levels (1.47-fold) of SREBF1 than the Alentejana bulls. The low silage diet promoted the *SREBF1* up-regulation (1.64-fold) in comparison to the high silage diet (P=0.028). Concerning the *ACACA* mRNA levels, while the Barrosã bulls fed the low silage diet tended to have higher expression levels than the high silage fed ones (2.24-fold), no variation between dietary treatments were found for the Alentejana bulls (breed×diet, P=0.082). A similar pattern was observed for the *PPARA* gene expression levels (1.6-fold, breed×diet, P=0.061). As for the *FABP4* gene expression, higher expression levels were found in the low silage fed Barrosã bulls when compared to those fed the high silage diet (1.55-fold), whilst in the Alentejana bulls there were no variations between feeding regimens (breed×diet, P=0.022).

Table 4.2 – Total lipids (g/100 g adipose tissue) and fatty acid composition (g/100g fatty acids) of subcutaneous adipose tissue from Alentejana and Barrosã breeds fed either high (HS) or low silage (LS) diets[1]

	Alentejana				Barrosã				*P*		
	HS		LS		HS		LS		Breed	Diet	Breed×Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total lipids	58.44	1.748	59.67	1.063	63.89	1.729	65.17	1.689	0.002	0.434	0.987
Main individual fatty acids											
14:0[§]	3.50	0.198	3.52	0.189	2.98	0.130	3.23	0.164	0.024	0.426	0.517
16:0[§]	27.24	0.429	26.57	0.552	23.63	0.444	25.11	0.659	<0.001	0.449	0.051
16:1*c*9[§]	4.03	0.380	3.83	0.242	4.89	0.263	5.41	0.248	<0.001	0.590	0.218
*a*17:0[§]	1.30	0.026	1.13	0.031	1.45	0.035	1.35	0.038	<0.001	<0.001	0.315
18:0	14.53	0.776	12.94	0.804	11.44	0.713	9.75	0.658	<0.001	0.034	0.946
18:1*t*11	1.41	0.123	1.36	0.129	1.88	0.125	1.74	0.113	0.002	0.456	0.705
18:1*c*9[§]	33.5	1.019	34.65	0.835	37.43	0.752	37.18	0.872	<0.001	0.610	0.431
18:1*c*11	4.13	0.396	3.7	0.367	4.32	0.144	4.53	0.142	0.093	0.692	0.279
CLA (*c*9*t*11)	0.40	0.034	0.34	0.026	0.80	0.041	0.81	0.041	<0.001	0.504	0.324
18:2*n*-6	1.49[b]	0.030	2.29[c]	0.141	1.70[a]	0.050	1.82[a]	0.064	0.181	<0.001	0.002
Partial sums											
Σ SFA	46.89	1.000	44.76	1.303	39.61	1.035	39.21	1.184	<0.001	0.273	0.453
Σ MUFA	44.89	1.034	46.00	1.323	50.62	1.092	51.54	1.223	<0.001	0.395	0.933
Σ TFA	2.73	0.181	3.57	0.277	3.75	0.176	3.79	0.145	0.004	0.037	0.057
Σ PUFA	1.80[a]	0.101	2.58[c]	0.144	2.07[b]	0.055	2.11[b]	0.067	0.347	<0.001	0.001
Σ *n*-3 PUFA	0.27[a]	0.012	0.26[a]	0.016	0.32[b]	0.008	0.25[a]	0.005	0.081	<0.001	0.012
Σ *n*-6 PUFA	1.53[a]	0.090	2.32[c]	0.134	1.75[b]	0.049	1.86[b]	0.065	0.216	<0.001	0.001
Σ BCFA	2.69	0.085	2.06	0.051	2.77	0.068	2.25	0.060	0.051	<0.001	0.411

[1] Different superscripts differ at least $P<0.05$. SFA = sum of 12:0,14:0, 15:0, 16:0, 17:0, 18:0 and 20:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 18:1*c*12, 18:1*c*13, 18:1*c*15, 19:1 and 20:1*c*11; TFA = sum of 18:1*t*6-*t*8, 18:1*t*9, 18:1*t*10, 18:1t11, 18:1t12, 18:1*t*16+*c*14, 18:2*c*9*t*11 and 18:2*t*11*c*15; PUFA = sum of 18:2*n*-6, 18:3*n*-3, 20:3*n*-6 and 20:4*n*-6; BCFA = sum of *i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0 and *i*18:0

[§] Variable adjusted for total lipids

4.3.4 Gene expression in muscle

The relative mRNA expression levels of the nine lipid metabolism key factors analysed in the LL muscle are presented in Figure 4.2. The CPT1B encoding gene tended to have higher expression levels (1.18-fold) in the Barrosã breed when compared to the Alentejana bulls (*P*=0.057). The *PPARG* gene showed a similar tendency (1.28-fold, *P*=0.081). The *ACACA* mRNA levels tended to be 1.19-fold higher in the low silage diet fed bulls when compared to the high silage fed ones (*P*=0.075). Conversely, the mRNA levels of *LPL* tended (*P*=0.083) to be higher (1.29-fold) in the high silage fed bulls than in their low silage fed counterparts. The *FABP4* gene showed higher expression levels (3.83-fold) in the low silage fed when compared to the high silage fed bulls (*P*=0.007), corresponding to a fold-change of 3.83. The expression levels of *CRAT*, *PPARA*, *SCD* and *SREBF1* genes were similar across experimental groups (*P*>0.05).

4.3.5 Correlation between gene expression and fatty acids

In order to elucidate the possible contribution of the lipogenic enzymes for fatty acid composition in SAT and muscle, the relationship between levels of gene expression and the content on main fatty acids was determined (Table 4.4).

For SAT, the correlation analysis revealed a positive relationship between total lipid content and *FABP4* gene expression level (*r*=0.38). In addition, this analysis showed negative correlations between this gene and 16:0 and 18:0 fatty acids (*r*=0.45 and *r*=0.41, respectively). Furthermore, positive correlations were found for *FABP4* expression level and 18:1*c*9 (*r*=0.34), 18:1*c*11 (*r*=0.37) and *c*9,*t*11 CLA (*r*=0.58), as well as for SFA (*r*=0.51) and MUFA (*r*=0.48). *LPL* gene expression had a negative association with 14:0 (*r*=-0.33) and 16:0 (*r*=-0.42), as well as the SFA (*r*=-0.34). In addition, positive correlations were found between *LPL* mRNA expression levels and total lipids (*r*=0.34), 18:1*c*9 (*r*=0.48) and *c*9,*t*11 CLA (*r*=0.35), as well as with MUFA (*r*=0.35). The *SCD* gene expression and the proportions of both 18:1*c*9 and *c*9,*t*11 CLA were positively correlated (*r*=0.51 and *r*=0.45, respectively).

Table 4.3 – Total lipids (g/100 g muscle) and fatty acid composition (g/100 g fatty acids) of *longissimus lumborum* muscle from Alentejana and Barrosã breeds fed either high (HS) or low silage (LS) diets[1]

	Alentejana				Barrosã				*P*		
	HS		LS		HS		LS		Breed	Diet	Breed×Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total lipids	1.21[a]	0.758	1.25[a]	0.836	1.76[b]	1.208	2.76[c]	1.955	<0.001	<0.001	0.001
Main individual fatty acids											
14:0†	2.04	0.082	2.24	0.121	2.29	0.075	2.71	0.098	<0.001	0.003	0.261
16:0†	23.50[a]	0.362	23.17[a]	0.439	23.33[a]	0.325	25.40[b]	0.373	0.011	0.028	0.003
16:1*c*9	2.37	0.162	2.52	0.102	2.85	0.103	3.27	0.189	<0.001	0.057	0.361
18:0	15.14	0.709	14.04	0.492	14.20	0.424	14.04	0.423	0.378	0.244	0.380
18:1*c*9‡	28.36	0.702	28.85	0.783	31.04	0.823	33.52	0.645	<0.001	0.053	0.189
18:1*c*11	2.90	0.302	3.35	0.083	3.40	0.254	3.08	0.088	0.586	0.762	0.078
CLA (*c*9*t*11)	0.21	0.011	0.20	0.014	0.45	0.025	0.45	0.018	<0.001	0.815	0.789
18:2*n*-6*	7.39[c]	0.387	8.32[c]	0.719	6.07[b]	0.329	4.22[a]	0.251	<0.001	0.333	0.007
20:4*n*-6†	2.39	0.156	2.25	0.148	1.53	0.140	1.28	0.117	<0.001	0.001	0.052
Partial sums											
Σ SFA†	42.10[a]	0.755	40.69[a]	0.815	41.29[a]	0.672	43.22[b]	0.624	0.209	0.736	0.037
Σ MUFA‡	35.77	0.876	36.92	0.866	41.58	0.825	40.31	0.939	<0.001	0.952	0.206
Σ TFA	1.65	0.095	2.03	0.145	2.49	0.106	2.62	0.114	<0.001	0.038	0.294
Σ PUFA*	11.92	0.465	12.57	0.929	9.11	0.959	7.29	0.491	<0.001	0.446	0.111
Σ *n*-3 PUFA†	1.50	0.095	1.13	0.059	1.23	0.134	0.661	0.038	<0.001	<0.001	0.288
Σ *n*-6 PUFA*	10.27	0.407	11.34	0.886	7.90	0.791	6.55	0.472	<0.001	0.843	0.083
Σ BCFA	1.66	0.062	1.41	0.057	1.71	0.054	1.46	0.054	0.394	<0.001	0.956

[1] Different superscripts differ at least $P<0.05$. SFA = sum of 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 21:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 18:1*c*12, 18:1*c*13, 18:1*c*15 and 20:1*c*11; TFA = sum of 18:1*t*6-t8, 18:1t9, 18:1t10, 18:1t11, 18:1 t16+c14 and 18:c9t11; PUFA = sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:3*n*-9, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3; BCFA = sum of *i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0 and *i*18:0 [2] Total lipids are expressed as g/100 g muscle; fatty acid composition is expressed as g/100 g fatty acids [3] HS: high silage; LS: low silage
† Variable adjusted for breed×total lipids; ‡ Variable adjusted for total lipids; * Variable adjusted for breed×diet×total lipids

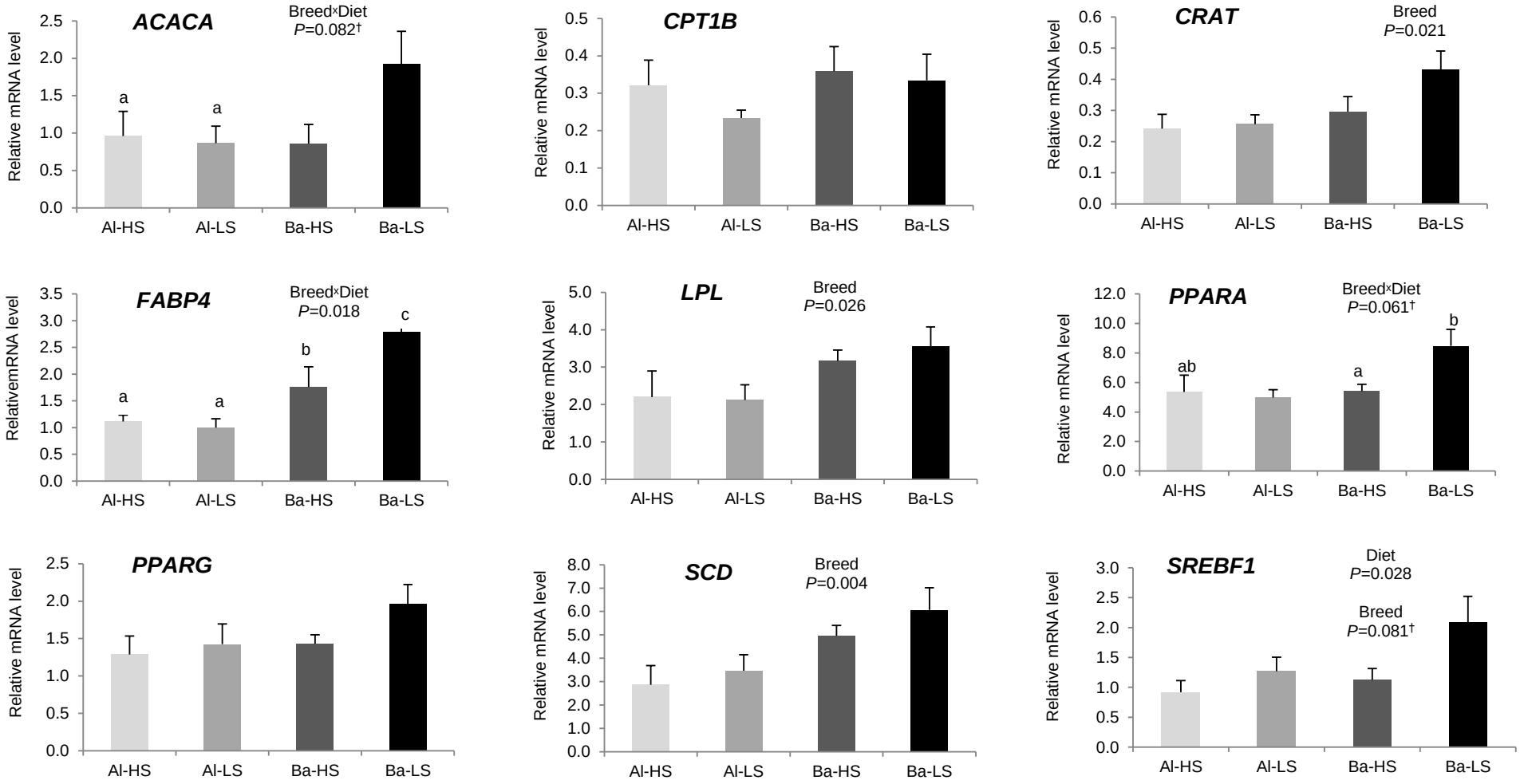

Figure 4.1 Relative expression levels of nine genes in the subcutaneous adipose tissue of Alentejana and Barrosã bulls fed high or low silage diets. Each value was normalized to *RPLP0* expression. Al-HS: Alentejana bulls fed the high silage diet; Al-LS: Alentejana bulls fed the low silage diet; Ba-HS: Barrosã bulls fed the high silage diet; Ba-LS: Barrosã bulls fed the low silage diet. Error bars indicate standard error. *FABP4* relative expression levels were adjusted for SAT total lipids content. †Tendencies were considered for 0.05<*P*<0.10. [a,b,c] Least square means with different superscripts differ at least *P*<0.05

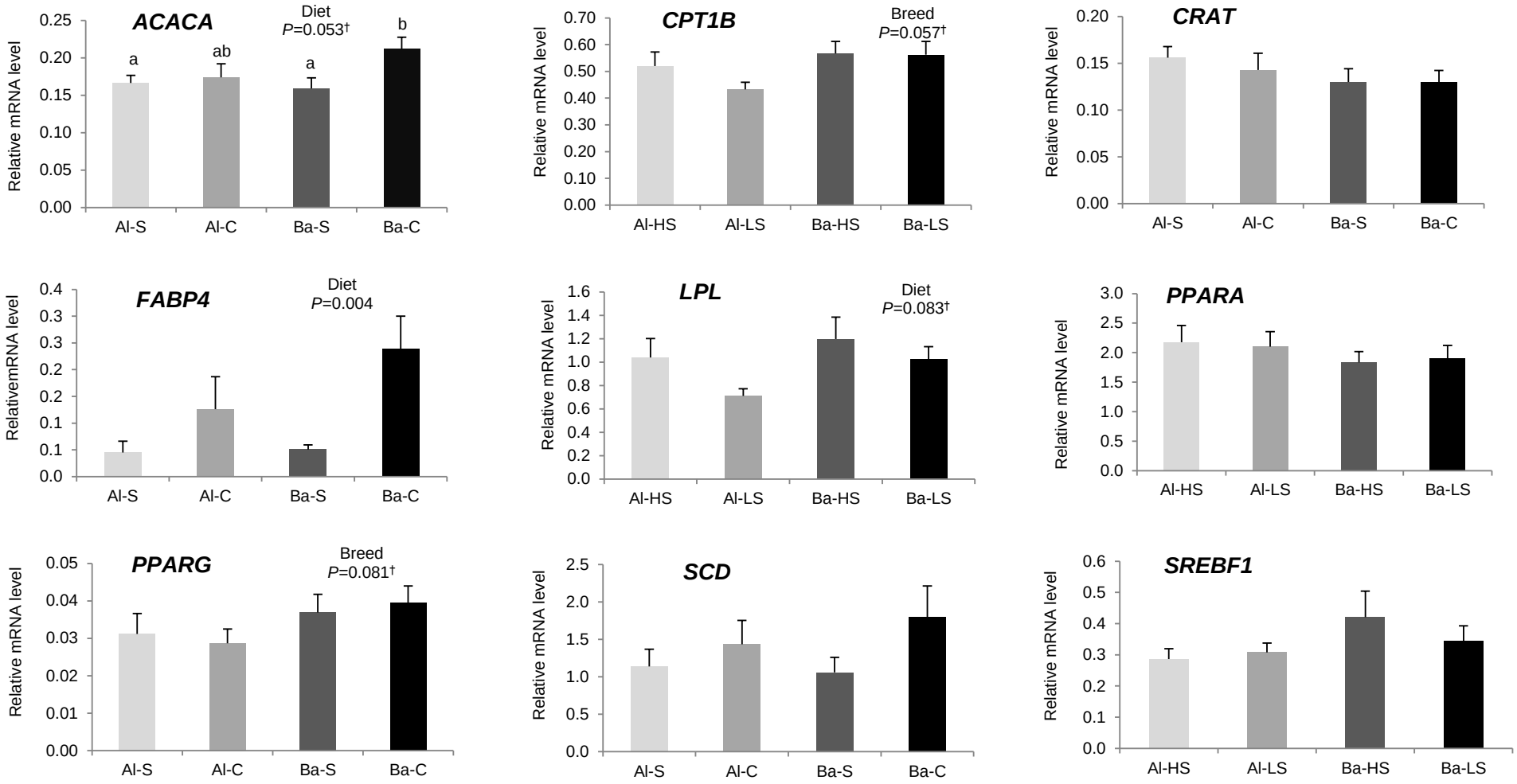

Figure 4.2 Relative expression levels of nine genes in the *longissimus lumborum* muscle of Alentejana and Barrosã fed high or low silage diets. Each value was normalized to *PPIB* expression. Al-HS: Alentejana bulls fed the high silage diet; Al-LS: Alentejana bulls fed the low silage diet; Ba-HS: Barrosã bulls fed the high silage diet; Ba-LS: Barrosã bulls fed the low silage diet. *ACACA* and *PPARG* relative expression levels were adjusted for total lipids content. *CRAT* relative expression levels were adjusted for breed×diet×total lipids content. *FABP4* relative expression levels were adjusted for diet×total lipids content. *SCD* relative expression levels were adjusted to breed×total lipids content. Error bars indicate standard error. †Tendencies were considered for 0.05<*P*<0.10

Table 4.4 – Pearson's correlation coefficients between genes expression levels and fatty acid composition in subcutaneous adipose tissue and *longissimus lumborum* muscle from Alentejana and Barrosã breeds[1-2]

	TL	14:0	16:0	18:0	18:1*t*11	18:1*c*9	18:1*c*11	CLA	SFA	MUFA	TFA
SAT											
ACACA	0.22	-0.10	-0.16	-0.11	0.09	0.26	-0.1	0.25	-0.17	0.19	0.06
CPT1B	-0.02	-0.04	-0.09	0.20	-0.04	-0.06	0.08	0.04	0.07	-0.05	-0.14
CRAT	0.21	-0.13	-0.25	-0.12	0.12	0.27	-0.15	0.26	-0.22	0.20	0.10
FABP4	**0.38***	-0.22	**-0.45****	**-0.41***	0.27	**0.34***	**0.37***	**0.58*****	**0.51*****	**0.48****	0.28
LPL	**0.34***	**-0.33***	**-0.42****	-0.16	0.15	**0.48****	-0.08	**0.35***	**-0.34***	**0.35***	0.07
PPARA	0.21	-0.10	-0.18	-0.14	-0.03	0.28	-0.11	0.20	-0.19	0.23	-0.05
PPARG	0.14	-0.20	-0.13	-0.12	-0.04	0.25	0.02	0.10	-0.18	0.21	0.02
SCD	0.24	-0.31	**-0.48****	-0.26	0.13	**0.51****	-0.03	**0.45****	**-0.44****	**0.43****	0.15
SREBF1	0.15	-0.19	-0.16	-0.19	-0.01	0.26	0.00	0.19	-0.23	0.24	0.08
Muscle											
ACACA	**0.48****	0.15	0.20	0.01	0.25	0.20	0.10	0.14	0.20	0.23	**0.40***
CPT1B	0.13	-0.05	-0.06	0.00	0.18	0.14	0.28	0.20	-0.11	0.21	0.05
CRAT	-0.17	-0.18	-0.13	0.20	-0.16	-0.11	0.17	-0.30	0.05	-0.10	**-0.33***
FABP4	**0.36***	0.19	0.20	-0.16	0.26	**0.42****	-0.11	0.17	0.07	**0.38***	0.21
LPL	0.09	-0.03	-0.05	-0.21	0.02	0.27	-0.06	0.14	-0.21	0.25	-0.13
PPARA	-0.19	0.08	0.17	-0.06	-0.30	**-0.33***	0.01	-0.21	0.09	-0.30	-0.27
PPARG	0.21	**0.35***	0.28	-0.27	0.17	0.22	-0.18	0.30	0.03	0.23	0.09
SCD	0.25	-0.05	0.04	0.08	0.26	0.13	0.14	0.13	0.19	0.12	**0.39***
SREBF1	0.08	0.14	0.16	-0.01	0.05	-0.14	**0.48****	0.31	0.13	0.03	**0.33***

[1]TL: total lipids; SAT: subcutaneous adipose tissue

[2] Significance level *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

A moderate association was also observed between *SCD* gene expression and MUFA (*r*=0.43). In addition, there was a negative correlation between 16:0 and expression of the *SCD* gene (*r*=-0.48). Concomitantly, SFA and *SCD* gene expression were also negatively correlated (*r*=-0.44).

Concerning the muscle, *ACACA* expression levels were correlated with total lipids (*r*=0.48), and the percentages of TFA (*r*=0.40). Correlations were also found between *FABP4* mRNA levels and total lipids (*r*=0.36), 18:1*c*9 (*r*=0.42), and MUFA (*r*=0.38). The mRNA levels for *PPARA* gene were negatively related to 18:1*c*9 (*r*=-0.33) percentages. Levels of *PPARG* expression were associated with the percentages of 14:0 *(r*=035). A positive association was found for *SCD* mRNA levels and TFA (*r*=0.39). Finally, 18:1*c*11 and TFA showed positive correlations (*r*=0.48 and *r*=0.33, respectively) with the expression levels of the *SREBF1* gene.

4.3.6 Principal Components Analysis

A Principal Components Analysis (PCA) was applied to a data set of fatty acid composition and gene expression parameters in order to describe the variability of the pooled data into two dimensions (Fig. 4.3A). The score plot of the first two components explains 70.1% of the total variability, with 57.1% for PC1 and 13.0% for PC2 (Table 4.5). In the score plot, there is a clear separation of most genes from the main fatty acids, except 18:1c9 and CLA (*c*9*t*11). Most of the genes were allocated on the right side of the plot (quadrant *b*), clearly separated from the other variables, showing positive scores for the PC1 and little influence on the PC2. In contrast, the *CPT1* gene was located on quadrant *a*, with positive scores for PC2. The PC1, which explains 57% of the variability, separated tissues (SAT *vs* muscle), with lipogenic genes and total lipids associated with SAT metabolism (quadrants *b* and *c*). The PC2, which explains 13% of the variability, separated palmitic (16:0) and myristic (14:0) fatty acids (quadrant *c*) from the remaining fatty acids. In quadrant *b*, a cluster was defined by *ACACA*, *CRAT*, *LPL*, *SCD*, *SREBF1* and *PPARA* genes. In addition, a cluster was formed by CLA, oleic acid (18:1*c*9) and the *FABP4* gene. The score plot depicted in Figure 4.3B shows the location of the LL muscle and SAT samples in the multivariate space of the first two PCs. These scores were notably arranged in two clusters, corresponding to each tissue. In contrast, no clear discrimination between breeds, Alentejana and Barrosã, or diet composition was achieved.

Table 4.5 - Loadings for the first three principal components

Variable	PC1	PC2	PC3
TL	0.883	-0.259	0.021
14:0	0.579	-0.681	0.260
16:0	0.274	-0.740	0.526
16:1*c*9	0.778	-0.319	-0.415
18:0	-0.592	0.060	0.643
18:1*c*9	0.762	-0.062	-0.327
CLA	0.723	-0.006	-0.492
18:2*n*-6	-0.784	0.443	-0.051
ACACA	0.799	0.339	0.297
CRAT	0.798	0.348	0.211
CPT1	-0.402	0.464	0.092
LPL	0.836	0.364	0.120
PPARA	0.862	0.212	0.215
PPARG	0.926	0.091	0.188
FABP4	0.853	0.080	-0.152
SCD	0.845	0.341	0.077
SREBF1	0.816	0.270	0.234
Proportion of variance (%)	57.10	13.04	9.43
Cumulative variance (%)	57.10	70.14	79.57

[1]PC: principal component; TL: total lipids

4.4 Discussion

The deposition of fat and fatty acids in animal tissues has been ascribed to a complex regulation network of lipogenic genes, although the molecular mechanisms underlying these systems remain to be established. Considering that adipose tissue physiology is related to both meat quality and animal production efficiency, understanding the factors affecting the depot-specific fat accretion and metabolism in beef cattle is of paramount importance. The present study addressed these aspects based on an experimental trial with two genetically diverse bovine breeds with distinct maturity rates, Alentejana and Barrosã, fed diets with different silage to concentrate ratios (30/70% *vs.* 70/30%). Albeit phylogenetically distant (Beja-Pereira *et al.*, 2003), Alentejana and Barrosã breeds are, nevertheless, more genetically similar than the breeds used in previous studies addressing adipogenic gene expression differences, mainly based on the Japanese Black genotype (Albrecht *et al.*, 2001; Taniguchi *et al.*, 2004; Wang *et al.*, 2009). Nonetheless, results reported here indicate that genetic background and, to a lesser extent diet composition, determine fat content and composition, pointing out to a differential fat partitioning between subcutaneous and intramuscular fat in Alentejana and Barrosã breeds.

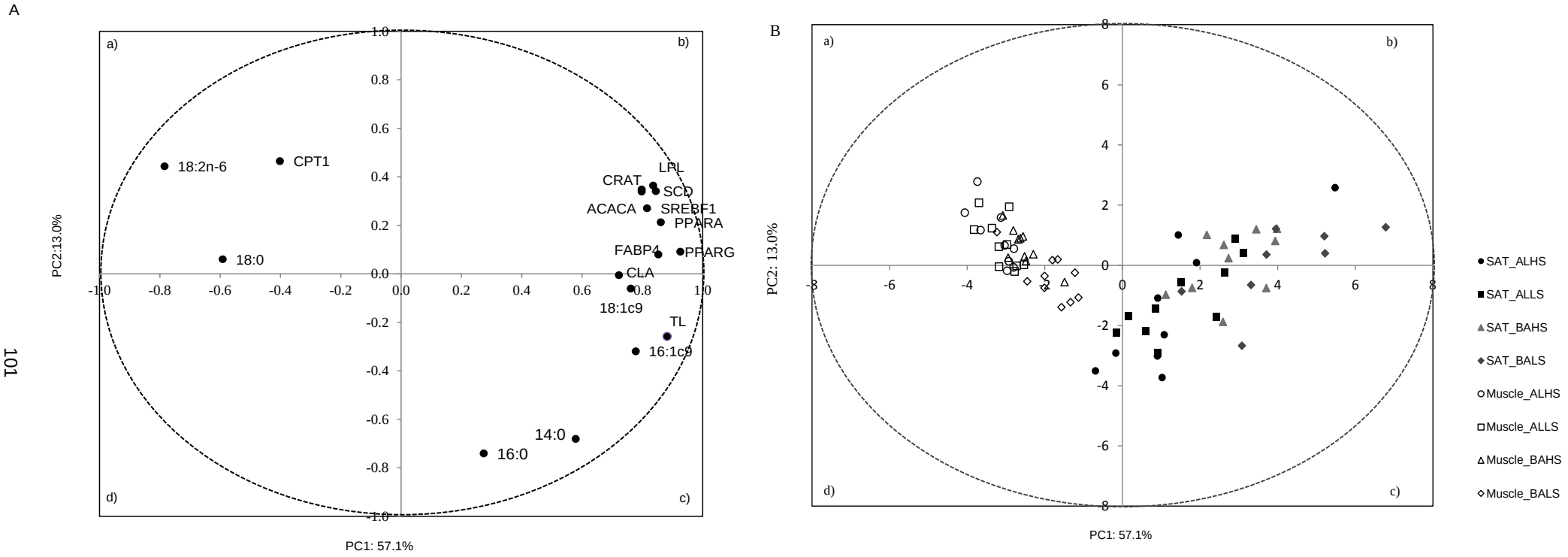

Figure 4.3 Loading plot of the first and second principal components (PC) of the pooled data (A) and component's score vectors (B) for *longissimus lumborum* muscle and subcutaneous adipose tissue from Alentejana and Barrosã bulls fed high or low silage diets. TL: total lipids; SAT: subcutaneous adipose tissue; Al-HS: Alentejana bulls fed the high silage diet; Al-LS: Alentejana bulls fed the low silage diet; Ba-HS: Barrosã bulls fed the high silage diet; Ba-LS: Barrosã bulls fed the low silage diet.

In order to elucidate the molecular mechanisms involved in this physiological process, these breed- and diet-specific variations are explained here through the transcript levels of nine key lipid metabolism-related genes. The PCA showed a close relationship among all adipogenic and lipogenic genes, which separated distinctively from *CPT1* gene, involved in the β-oxidation pathway, but not *CRAT*. PPARγ is a key activator of adipocyte differentiation and insulin sensitivity (Medina-Gomez *et al.*, 2007), inducing the transcription of many adipocyte genes encoding proteins and enzymes involved in the development and the maintenance of the adipocyte phenotype (Gregoire *et al.*, 1998). Surprisingly, levels of expression of *PPARG* were kept unchanged among experimental groups in the SAT samples, although Barrosã breed tended to have higher expression levels in the muscle than Alentejana bulls. It was expected that Barrosã breed, being a precocious breed , would had a greater proportion of adipocytes undergoing differentiation and, therefore, would had a greater relative expression of *PPARG* than Alentejana bulls. Although *PPARG* expression peaks during adipocyte differentiation, it is also expressed in mature adipocytes, but at lower levels (Scollan *et al.*, 2006). Harper and Pethick (2004) reported that the expression of *PPARG* decreases substantially as growth proceeds. Therefore, treatment differences may not have been detected if one group had adipocytes undergoing differentiation, whereas the other group may have greater number of mature adipocytes resulting in similar *PPARG* expression levels. Differences could, however, be detected at earlier stages of development. These results can be explained by the circumstance that subcutaneous fat develops earlier than intramuscular fat and, therefore, the tendency observed in the muscle was not detected in the SAT.

The *SREBF1* gene encodes a transcription factor involved in adipocyte differentiation (Briggs *et al.*, 1993) and in the biosynthesis of fatty acids (Brown & Goldstein, 1997) being, possibly, implicated in a mechanism that links adipogenesis and lipogenesis. In particular, the SREBF1c isoform appears to be primarily involved in regulating the expression of lipogenic and fatty acid-metabolizing enzymes. The transcription and activation of SREBF1c protein was proposed to be regulated by the degree of saturation of lipids (Worgall *et al.*, 1998). Graugnard *et al.* (2010) suggested that the expression of the *SREBF1* gene could be nutritionally regulated. Transcriptional regulation of *SREBF1* in most non-ruminant animals is sensitive to insulin, which under times of carbohydrate excess leads to stimulation of fatty acid synthesis and TAG deposition in adipose tissue (Kim *et al.*, 1998). In the present study, insulin levels were also the highest in low silage fed bulls, as were the expression levels of *SREBF1* in the SAT. Although the transcription factor *SREBF1* is considered to regulate *SCD* expression (Sekiya *et al.*, 2007), its expression levels were not consistent with that of *SCD* in the muscle.

As pre-adipocytes differentiate into mature adipocytes, *LPL* is one of the first genes expressed due to its role in the *de novo* fatty acid synthesis (Gregoire *et al.*, 1998). A higher relative *LPL* mRNA expression in the SAT could indicate that Barrosã bulls had more adipocytes undergoing differentiation when compared to Alentejana bulls. Pickworth *et al.* (2011) proposed that *LPL* may be a more definitive indicator of adipocyte differentiation than *PPARG.* Concerning the relationships between the fatty acids and *LPL* expression in SAT, the correlation analysis revealed close relationships between levels of *LPL* mRNA and 18:1*c*9, as well as *c*9,*t*11 CLA, among others. These results highlight the role of *LPL* in the control of TAG uptake and, consequently, on the fatty acid profile. However, the same pattern was not observed for the muscle and *LPL* showed no clear association with the main fatty acids. Taking into account that both *SCD* and *LPL* are expressed late in adipogenesis, increased gene expression would be consistent with more active adipocytes in SAT from Barrosã than in Alentejana bulls. Taken together, these results suggest that Barrosã bulls may have more differentiated adipocytes, which are capable of storing fat in the subcutaneous depot.

FABP4 protein is responsible for the transport of fatty acid outside the cell (Yang *et al.*, 2011) and plays a role in lipolysis and fatty acid trafficking in different tissues (Shen *et al.*, 1999; Damcott *et al.*, 2004; Cho *et al.*, 2008). Fat storage and metabolism within functional adipocytes are modulated by FABP4 (Gregoire *et al.*, 1998), and thus levels of expression of this gene can be used as a marker of fully differentiated adipocytes (Pickworth *et al.*, 2011). In fact, its association with intramuscular fat content and backfat thickness have been reported by Jurie *et al.* (2007). Results herein presented for *FABP4* expression are concomitant with a higher fatty acids deposition in the SAT from Barrosã when compared to Alentejana bulls. The higher expression levels of *FABP4* in Barrosã bulls fed the low silage diet may be indicative of a higher level of adipogenic differentiation in the SAT of these animals. Several studies reported an association between bovine *FABP4* gene expression or protein activity and intramuscular fat content (Briggs *et al.*, 1993; Fernyhough *et al.*, 2005; Jurie *et al.*, 2007), as well as backfat depth (Michal *et al.*, 2006). Accordingly, in this study, the Barrosã carcasses had the highest intramuscular fat and carcass fatness scores. Furthermore, levels of *FABP4* expression were moderately associated with total lipids contents in the muscle, as well as with the 18:1*c*9 proportions and the associated desaturation index. On the other hand, *FABP4* was associated with the main fatty acids in the SAT. These results reinforce the role of *FABP4* in intramuscular fat development and the SAT as the major site for lipid metabolism in ruminants.

ACACA is a key regulator of lipogenesis and the rate-controlling enzyme in adipose tissue of meat-producing animals (Laliotis *et al.*, 2010). The expression of the *ACACA* gene is highly inducible in the major lipogenic tissues (Laliotis *et al.*, 2010) and the enzyme is nutritionally

regulated (McGarry *et al.*, 1989; Joseph *et al.*, 2010). In a study by Joseph *et al.* (2010), it was found that oleic acid had an inhibitory effect on the expression of lipogenic genes in the SAT. In line with this, data herein presented revealed that, in SAT, *ACACA* expression levels tended to be the highest in Barrosã bulls fed the low silage diet (lowest oleic acid content). However, in the muscle, the expression levels tended to be lower in the high silage fed animals. These results suggested that the effect of dietary fatty acid composition is not only influenced by the genetic background but also by the fat depot location. In addition, *ACACA* mRNA levels were shown to have a positive association with total lipid content in the muscle samples, thus adding evidence to the importance of this enzyme to intramuscular fat deposition in ruminants.

Dietary *n*-3 and *n*-6 PUFA have been shown to inhibit *de novo* lipogenesis in dairy cattle (Chilliard *et al.*, 1991). Accordingly, our results showed a tendency for higher *ACACA* expression levels in the muscle from the low silage- in comparison to the high silage-fed bulls. Underwood *et al.* (2007) reported a positive relationship between ACACA enzyme activity and intramuscular fat, which is consistent with our findings. In the present study, total lipids in the muscle were positively related to the expression of *ACACA*. Although this gene codifies for an enzyme which catalyses the formation of SFA, there was a positive association between *ACACA* expression levels and TFA. This suggests that an increase in TFA biosynthesis, that is, in desaturase activity, is a major factor in intramuscular (but not SAT) deposition in ruminants. In addition, *CPT1B* tended to show lower expression levels in the muscle from Alentejana, indicating higher fatty acid oxidation in the Barrosã breed.

Results concerning *SCD* gene expression, considered a marker of mature adipocytes (Graugnard *et al.*, 2010), support the concept of a higher degree of maturity of the SAT adipocytes from the Barrosã than the Alentejana bulls, as observed for the *FABP4* gene. The fatty acid composition of SAT mirrors the action of the SCD protein on substrates like stearic and palmitic acids. The correlation analysis between oleic acid proportions and *SCD* expression levels in the SAT was showed to be positive and significant. This result is in agreement with previous reports (Daniel *et al.*, 2004) that reported that increased SCD activity is, at least partially, responsible for an elevated oleic acid, the main MUFA, content in ruminant. Similarly, a significant correlation was also found between *c*9,*t*11 CLA percentage and *SCD* expression levels. Taniguchi *et al.* (2004) reported a positive correlation between levels of *SCD* mRNA and MUFA proportion, which led to the conclusion that *SCD* expression might contribute to the differences in the SAT fatty acid composition between breeds. The variations observed in the fatty acid classes in the SAT and muscle samples were mostly due to breed. High MUFA proportions in SAT have been reported (Lehnert *et al.*, 2007) and might be related to an elevated SCD activity. Overall, our results support the concept of a central role for *SCD* in adipose tissue fatty acid synthesis. Genes encoding lipogenic enzymes

responsible for the *de novo* SFA synthesis and MUFA production were down-regulated in Alentejana breed when compared to Barrosã bulls. This is in agreement with changes in tissue fatty acid composition, in which 16:0 concentration, the end product of *de novo* SFA synthesis, and *c*9,*t*11 CLA, a product of Δ^9 desaturation, were reduced in the SAT of Alentejana bulls when compared to that of Barrosã animals.

In contrast to what was observed in SAT, the expression levels of the *SCD* gene in the muscle were similar among the four experimental groups. Several authors reported that *SCD* mRNA (Briggs *et al.*, 1993) or protein expression levels (Ward *et al.*, 2010) in muscle do not reflect intramuscular fat levels. The results herein reported are consistent with those of the previous studies. In addition, an association between *SCD* mRNA levels and MUFA contents in bovine SAT has been reported in several works (Chung *et al.*, 2007; Dance *et al.*, 2009). Nonetheless, the same association has not been reported for muscle (Ward *et al.*, 2010; Dance *et al.*, 2009; Bartŏn *et al.*, 2011), which could point to a depot-specific regulation mechanism of *SCD* gene expression and/or enzyme activity. Finally, it should also be noted that there was a high individual variation in the *SCD* mRNA levels. Therefore, despite changes in relative gene expression mirroring the changes in MUFA proportion, the correlation analysis failed to establish a significant association between both. The lack of association between *c*9,*t*11 CLA and *SCD* expression is in agreement with data reported by both Ward *et al.* (2010) and Bartoň *et al.* (2011). Positive correlations, however, were found between the levels of *SCD* gene expression and total lipids, as well as TFA, both of which increase as fattening proceeds (Ntambi & Miyazaki, 2004). It should however be taken into account that conclusions drawn from single-point observations may lead to the erroneous assumption that *SCD* was not affected and/or had a crucial role in intramuscular fat synthesis. In that regard, Graugnard *et al.* (2010) suggested that responses to high starch diets might not necessarily lead to increased adipogenesis. Moreover, these authors reported with some surprise that animals fed a low starch/high fibre diet during the growing phase showed increases in expression of lipogenic genes *PPARG*, *FABP4*, and *SCD* during the finishing phase.

The *PPARA* gene induces the expression of the fatty acid β-oxidation genes (Barbier *et al.*, 2002; Nakamura & Nara, 2003). The tendency for a higher expression of *PPARA* in the SAT from Barrosã bulls fed the low silage diet in comparison to the remaining experimental groups is concomitant with a more intense β-oxidation in the former breed. In addition, PPARα has been shown to induce the expression of Δ^5 and Δ^6 desaturase genes (Guillou *et al.*, 2002). However, results from the present study revealed no clear association between the *PPARA* mRNA levels and the fatty acid composition of SAT.

Carnitine acyltransferases catalyse the exchange of acyl groups between carnitine and coenzyme A (CoA) (Jogl *et al.*, 2006). These enzymes include CRAT and carnitine

palmitoyltransferases (CPTs) (Jogl *et al.*, 2006). The CPTs transesterify medium and long chain fatty acyl chains, whereas CRAT transesterifies short-chain acyl chains (Calabrese *et al.*, 2006). High *CRAT* gene transcription levels may be indicative of an elevated number of differentiating cells during growth (Jogl *et al.*, 2006). The higher *CRAT* transcription levels found in the Barrosã bulls are in agreement with the higher lipid accumulation in the Barrosã SAT, in comparison to the Alentejana bulls. In contrast, the lack of a significant variation in their relative expression levels of both genes' in muscle samples is in agreement with the similar expression levels observed for the genes indicative of terminal adipocyte differentiation (*SCD*, *LPL* and *PPARG*). In addition, the close association between *CRAT* and the genes involved in lipogenesis reinforces the relationship between lipogenesis and β-oxidation, thus being indicative of high lipid turnover in those animals with high lipid deposition.

If in SAT there was a clear effect of breed in fatty acid deposition, with no influence of diet composition, in the LL muscle the response to dietary silage levels depended on animals' genetic background. In previous studies (Smith & Crouse, 1984; Schoonmaker *et al.*, 2004), diets differing in starch contents (high starch *vs*. low starch), which is also the case of the present study, resulted in higher intramuscular fat in animals fed high starch diets. The interaction between breed and diet found suggests that the concept that high concentrate diets increase beef intramuscular (Aldai *et al.*, 2007) may be determined by breed and/or maturity. The differential response to diet composition between Alentejana and Barrosã bulls suggests distinct ruminal biohydrogenation patterns, as indicated by the intramuscular fatty acids contents, as well as the increased 18:2*n*-6 metabolic availability in Alentejana bulls fed the low silage diet. A differential regulation of fatty acid metabolism in distinct fat depots could explain the fact that no similar response was observed in SAT. Indeed, various fat depots have been reported to differ markedly in lipogenic activity (Eguinoa *et al.*, 2003; Dodson *et al.*, 2010). An increase in the SFA content is to be expected whenever the concentrate proportion in the diet is increased. Feed silage level had no effect on total SFA of both tissues. However, in the SAT stearic acid was higher in high silage than in low silage fed bulls, and also higher in Alentejana when compared to Barrosã breed, but no such effects were found in the LL muscle. Similarly, both total and individual MUFA were not responsive to dietary silage level in the SAT, whereas in the LL muscle a tendency was found for the low silage diet to promote higher percentages of these fatty acids. Both PUFA and TFA were influenced by breed and diet, but the response to breed and diet factors differed between tissues. The BCFA were the only fatty acids showing similar response to diet, given that they originate ruminal activity and suffer no further modifications until being deposited in tissues. Taken together, these results suggest a differential regulation of fatty acid metabolism between tissues, possibly resulting from the contrasting roles of

intramuscular and subcutaneous fat depots. In addition, the data also indicate differences in genetic background reflected in the response to diet composition, both in the gene expression of adipogenic and lipogenic factors and fatty acid composition of tissues.

The PCA confirmed the tissues contrasting features regarding lipid metabolism and fatty acid composition, showing a clear separation between the muscle and the SAT, as was previously shown by the analysis of variance. In addition, this statistical approach showed that there is less variability in muscle fatty acid composition and gene expression when compared to the SAT, as depicted by the plot of component scores. Furthermore, the PCA indicates that the expression levels of most adipogenic and lipogenic genes, along with linoleic acid, are the variables with the most discriminant power between tissues.

4.5 Conclusions

The results herein presented suggest that, at 18 months old, Barrosã bulls might have more differentiated adipocytes and lipids deposited in SAT than Alentejana animals. Moreover, both lipogenesis (*SCD* and *LPL*) and β-oxidation (*CRAT*) related genes had higher levels of mRNA in the SAT from Barrosã animals when compared to Alentejana bulls. These data indicate a higher storage/removal ratio of TAG and a greater dynamics of lipid turnover in the SAT of Barrosã breed relative to Alentejana bulls.

The fatty acid deposition in the SAT is mainly influenced by dietary silage level, whereas the effect of breed is mostly associated with the expression level of the transcription factor *SREBF1*. Combined effects of breed and diet were obtained for the *de novo* fatty acid synthesis (*ACACA*) and fatty acid transport in adipocytes (*FABP4*) related genes, and the transcription factor *PPARA* mRNA levels.

In contrast to SAT, only a slight breed effect was obtained for muscle, with the expression levels of *PPARG* and *CPT1B* showing a tendency to be higher in Barrosã bulls. However, the low silage diet, relative to the high silage diet, increased the levels of *FABP4* and *ACACA* mRNA and tended to decrease *LPL* expression in the muscle.

Taken together, the results herein presented show that lipid metabolism in SAT is more sensitive to breed than muscle, whereas lipid metabolism in the latter tissue appears to be mostly diet-dependent. The differential gene expression patterns in SAT and muscle are likely responsible for the fatty acid partitioning between both tissues, thus reinforcing the prevailing role of SAT over intramuscular fat in the *de novo* fatty acid synthesis. These findings provide evidence for breed- and tissue-specific variations in fatty acid content and composition of beef cattle, which can be explained, at least in part, by the expression of key adipogenic and lipogenic genes involved in lipid metabolism. This insight into the molecular mechanisms underlying fat deposition in bovine SAT and muscle in different breed may

contribute to the development of diet-based strategies to improve competitiveness of beef industry in order to satisfy consumers' expectations.

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CHAPTER 5

Meat fatty acid composition

Genetic background and diet impact beef fatty acid composition

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ASH Costa collaborated in the sample collection, performed the laboratory work and data processing and was responsible for the statistical analysis. In addition, ASH Costa participated in the interpretation and discussion of the results, as well as in the writing the manuscript.

Abstract

Intramuscular fat composition of ruminant meats influences the quality of the final product, which explains the increasing interest in assessing the fatty acid profile of meat from different production systems. In this study, it was hypothesized that there are breed- and diet-induced variations on lipid metabolism in the muscle which may be, at least partially, modulated by the *SCD* gene expression levels. Forty purebred young bulls from two phylogenetically distant autochthonous cattle breeds, Alentejana and Barrosã (n=20 for each breed), were assigned to two different diets (low *vs*. high silage) and slaughtered at 18 months of age. Meat fatty acid composition, including the detailed CLA isomeric profile, was determined. Meat from Barrosã bulls fed the low silage diet was richer in monounsaturated fatty acids, CLA and TFA, when compared to that from Alentejana bulls. The meat content in polyunsaturated fatty acids was similar across experimental groups. Overall, these findings highlight the importance of taking into account the genetic background while devising feeding strategies to manipulate beef fatty acid composition.

5.1 Background

Fat content and fatty acid composition of meat-producing animals has received considerable attention due to its implications for meat nutritional, organoleptic and technological traits (Wood *et al.*, 2008). Meat from ruminants has been shown to provide high levels of SFA and TFA, but low levels of PUFA (Hocquette *et al.*, 2010). Modern-day consumers are health conscious and, therefore, concerned about fat composition, as scientific evidence suggests distinct effects of fatty acids on human health. In line with this, the current nutritional recommendations strongly encourage a reduction in the intake of SFA and TFA, and an increase in the intake of *n*-3 PUFA, in particular EPA (20:5*n*-3) and DHA (22:6*n*-3) (Burlingame *et al.*, 2009; EFSA, 2010). In addition, a multitude of potential health benefits have been attributed to some conjugated isomers of linoleic acid, found in meat and milk from ruminant animals, commonly known as CLA (Prates & Bessa, 2009). However, specific physiological effects have been linked to individual CLA isomers, thus implying the determination of the CLA isomeric profile in ruminant-derived fat. The *t*10,*c*12 isomer may play an important role in lipid metabolism, while the *c*9,*t*11 and the t10,c12 isomers seem to be equally effective in anticarcinogenesis (Park & Pariza, 2007)in animal models. These beneficial effects in humans are controversial (Benjamin & Spener, 2009; Kennedy *et al.*, 2010).

Contrary to monogastric animals, ruminants do not deposit tissue fatty acids in direct proportion to dietary lipid composition due to ruminal hydrolysis of acylglycerols and extensive hydrogenation (from 86 to 95%) of the dietary PUFA (Scollan *et al.*, 2001a). It is well documented that the intramuscular fat content and composition in bovines is strongly influenced by the feeding system, age and slaughter weight (Moreno *et al.*, 2006). Works by Harper and Pethick (2004) and Scollan *et al.* (2006) proposed that genetic variation in fatty acid metabolism among breeds impacts on fatty acid composition. In addition, changes in beef cattle fatty acid composition have been reported in relation to the type and level of dietary fat (Vatansever *et al.*, 2000), energy level (Scollan *et al.*, 2001b) and forage to concentrate ratios in the finishing diet (De Smet *et al.*, 2000). However, while feeding strategies to modify fatty acid composition have been extensively studied, there is still much to be unveiled regarding the genetic factors involved (De Smet *et al.*, 2004).

It has been suggested that the lipogenic enzyme SCD, which catalyses the rate-limiting step of MUFA biosynthesis, plays a key role in fat deposition (Taniguchi *et al.*, 2004) The major CLA isomer, *c*9,*t*11, is produced in the rumen during the microbial biohydrogenation of dietary 18:2*n*-6 and endogenously through desaturation of the 18:1*t*11 by SCD (Griinari & Bauman, 1999). Furthermore, the *SCD* mRNA in muscle was reported to increase during the

late fattening stages (Kwon *et al.*, 2009) in a breed- and diet-dependent manner (Taniguchi *et al.*, 2004; Chung *et al.*, 2007).

Dietary manipulations in order to decrease the content of SFA and increase the levels of *n*-3 PUFA and CLA in ruminant meats have been widely used. However, a detailed determination of CLA and TFA isomers is necessary, as dietary manipulations affect distinctly the profile of these fatty acids and the individual isomers have specific biological effects. In addition, the response to diet composition may depend on the genetic background. Alentejana, a large-framed bovine breed from the South of Portugal, and Barrosã, a small-framed bovine breed from the Northwest of Portugal, are phylogenetically distant (Beja-Pereira *et al.*, 2003), thus possessing distinct genetic backgrounds. Therefore, these two autochthonous beef cattle breeds (Alentejana and Barrosã) and two different diets (based on 30/70% and 70/30% of maize silage and concentrate, respectively) were selected to clarify the breed and diet induced variations on the fatty acid composition and *SCD* gene expression levels. It was, therefore, hypothesised that lipid metabolism in muscle may be modulated by the genetic background and diet composition, thus influencing the *SCD* gene expression level in muscle and meat fatty acid composition. To test this hypothesis, the detailed fatty acid composition of total lipids and their polar and neutral fractions of meat from Alentejana and Barrosã bulls was determined, in parallel to the assessment of muscle *SCD* gene mRNA relative expression levels.

5.2 Materials and Methods

5.2.1 Animals and experimental design

Details concerning the experimental design, animals and the composition of experimental diets are provided in chapter 2, section 2.2.

5.2.2 Muscle and meat sampling

For gene expression analysis, LL muscle samples were collected and processed as described in chapter 4, section 4.2. Details the maturation of the carcass and separation into commercial joints can be found in chapter 2, section 2.2. The LL muscle samples (*ca*. 200 g) were collected, trimmed of connective and adipose tissues before being blended in a food processor, vacuum packed and stored at –20 ºC until lipid analysis.

5.2.3 Lipid extraction and methylation

Meat samples were lyophilized (–60 °C and 2.0 hPa) and maintained exsiccated. Intramuscular lipids were extracted by the method of Folch *et al.* (1957), as described in chapter 2, section 2.2. The lipid extract was separated into NL and PL fractions, using the solid-phase extraction procedure described by Juaneda and Rocquelin (1985) and silica gel cartridges (LiChrolut_Si, 40–63 lm, 500 mg/ml, Standard, Merck KGaA, Darmstadt, Germany). The NL fraction was eluted with dichloromethane and the PL fraction with methanol.

5.2.4 Lipid analysis

5.2.4.1 Preparation of FAME and gas chromatography analysis

The total, NL and PL of muscle were transesterified with sodium methoxide followed by hydrochloric acid in methanol and the resulting FAME were then analysed by GC, as described in chapter 3, section 3.2.5. The same FAME solution was used for the analysis of both fatty acid composition and CLA isomeric profile, enabling the direct comparison of quantitative data and eliminating differences in sample preparation. It should be noted that the peaks of 18:1*c*9 and 18:1*c*11 might include minor amounts of *t*12, *t* 13, *t*14, *t*15, *c*6, *c*7, *c*8 and 18:1*c*10 isomers.

5.2.4.2 Ag+-HPLC analysis

CLA isomers were individually separated by triple silver-ion columns in series using a HPLC system, as described in chapter 3, section 3.2.5.

5.2.5 Gene expression analysis by real-time reverse transcription quantitative PCR

The detailed procedures concerning RNA extraction, cDNA synthesis, primer sequences for *SCD* and *PPIB* genes, and overall RT-qPCR are described in Chapter 4.

5.2.6 Statistical analysis

For statistical analysis, the SAS software version 9.2 (SAS Institute Inc., 2009) was used. All statistical analyses were performed based on a 2 × 2 factorial arrangement of breed (Alentejana and Barrosã purebreds), diet (HS and LS diets) and their interaction. The variances were tested for heteroscedasticity and, for most variables, variance was found to be heterogeneous. Therefore, subsequent data analysis was performed in order to account for heterogeneous variance. The general Satterthwaite approximation was computed in a mixed-effects regression model (PROC MIXED; SAS Institute Inc., 2009), with breed, diet and their interaction as fixed effects.

Results were expressed as mean ± standard error. Differences between groups were examined for statistical significance using the PDIFF option (Fisher's test). Differences were significant at $P<0.05$ and tendencies discussed at $P<0.10$. Pearson correlation coefficients were calculated using the CORR procedure of SAS.

5.3 Results

5.3.1 Intramuscular fatty acids

Total fatty acid concentration in the muscle PL fraction (Table 5.2) did not differ among experimental groups ($P>0.05$). In addition, total fatty acid concentration in the muscle NL fraction (Table 5.3) was higher in Barrosã bulls fed the low silage diet, intermediate in those fed the high silage diet, and lower in Alentejana bulls fed with any of the diets (breed×diet, $P<0.001$). Consistently, the concentration of total lipids (breed×diet, $P<0.001$) and total fatty acids (breed×diet, $P<0.001$) in meat (Table 5.4) followed the same pattern as total fatty acids in the NL fraction.

5.3.2 Meat polar fatty acids

The main fatty acid percentages (mol%), as well as the stearoyl-CoA (Δ^9) desaturation indices, in the PL fraction of muscle are shown in Table 5.2. Meats from Barrosã bulls had higher percentages of 18:1*t*9 ($P<0.05$), 18:1*t*11 ($P<0.01$), co-eluted 18:1*t*16 plus -*c*14 ($P<0.01$) and 20:4*n*-6 ($P<0.05$), when compared to those from Alentejana bulls. In turn, Alentejana bulls had higher 16:0 ($P<0.01$) and 18:1*c*12 ($P<0.05$) when compared to Barrosã bulls.

Feeding the high silage diet promoted the deposition of 14:1*c*9, 18:0, 18:1*t*16 plus -*c*14, 18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3 in comparison to the low silage diet ($P<0.05$). Higher percentages of 18:1*c*11 and 20:2*n*-6 were found in the bulls fed the low silage diet when compared to those fed the high silage diet ($P<0.001$). The low silage diet promoted lower proportions of 16:1*c*9, 18:1*c*9 and 20:3*n*-9 than the high silage diet in Alentejana bulls, whereas a similar response to both diets was found for Barrosã bulls (breed×diet, $P<0.01$). Conversely, the Alentejana bulls fed the low silage diet had the highest 18:2*n*-6 and 20:3*n*-6 percentages (breed×diet, $P<0.05$). The Barrosã bulls had the highest CLA percentages when fed the high silage diet (breed×diet, $P<0.05$). The Δ^9-index for 16:0 was lowest in Alentejana bulls fed the low silage diet (breed×diet, $P<0.001$). On the other hand the desaturation index for 18:0 was highest in Barrosã bulls fed the low silage diet (breed×diet, $P<0.01$).

5.3.3 Meat neutral fatty acids

The percentages of the main fatty acids, as well as the Δ^9 desaturation indices, in the NL fraction of muscle are shown in Table 5.3. Barrosã bulls had higher 14:1*c*9, 16:1*c*9, 18:1*t*11, 18:1*c*9, 18:1*c*12, 18:1*t*16 plus -*c*14 and total CLA than Alentejana bulls ($P<0.05$). In contrast, 18:0 and 18:1*t*6 to *t*8 were higher in Alentejana than in Barrosã bulls ($P<0.01$). In addition, the indices for Δ^9 desaturase activity were consistently higher in Barrosã than in Alentejana bulls ($P<0.05$). The high silage diet promoted a higher deposition of 18:1*t*16 plus -*c*14 and 18:3*n*-3 when compared to the low silage diet ($P<0.05$).

Alentejana bulls fed the low silage diet had the highest percentages of 16:0 and 18:1*c*13 (breed×diet, $P<0.01$). In Alentejana bulls, the low silage diet promoted higher percentages of 18:1*t*10 than the low silage diet, whereas in Barrosã bulls the increase in this fatty acid was not as expressive (breed×diet, $P<0.01$). In addition, feeding the low silage diet to the Alentejana bulls increased the percentage of 18:2*n*-6 when compared to the high silage diet, whereas the same effect was not observed for the Barrosã bulls (breed×diet, $P<0.05$). Barrosã bulls fed the low silage diet had the highest percentages of 18:1*c*11 when compared to those fed the high silage diet, whereas in Alentejana bulls diet had a less pronounced effect on the deposition of this fatty acid (breed×diet, $P<0.05$). The 20:4*n*-6 contents were increased by the low silage diet in Alentejana bulls but not in Barrosã bulls (breed×diet, $P<0.05$).

Table 5.1 – Fatty acid concentration (mg/g fresh muscle) and centesimal distribution (mol% of total fatty acids) of fatty acids in polar lipids of the *longissimus lumborum* muscle from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets.

	Alentejana				Barrosã				*P* value		
	HS		LS		HS		LS		Breed	Diet	Breed×Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total fatty acids	3.54	0.24	2.82	0.20	4.10	0.85	3.93	0.26	0.083	0.345	0.569
Fatty acids											
14:0	0.55	0.07	0.37	0.04	0.50	0.08	0.46	0.08	0.770	0.119	0.337
14:1*c*9	0.21	0.01	0.18	0.01	0.22	0.02	0.18	0.02	0.849	0.012	0.739
16:0	20.2	0.43	20.3	0.53	18.3	0.44	19.0	0.33	0.001	0.381	0.549
16:1*c*9	1.41^{b}	0.12	0.90^{a}	0.06	1.19^{b}	0.07	1.42^{b}	0.09	0.105	0.125	<0.001
18:0	7.71	0.25	6.99	0.39	7.84	0.26	7.06	0.15	0.722	0.012	0.912
18:1*t*6-*t*8	0.18	0.02	0.17	0.00	0.11	0.01	0.21	0.02	0.155	0.612	0.208
18:1*t*9	0.09	0.01	0.09	0.00	0.09	0.00	0.12	0.01	0.026	0.278	0.083
18:1*t*10	0.08^{a}	0.00	0.15^{b}	0.02	0.08^{a}	0.00	0.11^{b}	0.01	0.142	<0.001	0.044
18:1*t*11	0.27	0.02	0.26	0.02	0.38	0.02	0.32	0.02	<0.001	0.138	0.256
18:1*c*9	21.3^{b}	1.36	16.5^{a}	0.78	18.6^{ab}	0.97	19.8^{b}	0.76	0.809	0.077	0.006
18:1*c*11	2.44	0.05	2.89	0.12	2.45	0.06	2.82	0.12	0.745	<0.001	0.705
18:1*c*12	0.63	0.04	0.67	0.04	0.54	0.02	0.57	0.03	0.011	0.385	0.798
18:1*c*13	0.11	0.01	0.12	0.01	0.11	0.01	0.14	0.01	0.295	0.065	0.354
18:1*c*14+*t*16	0.09	0.00	0.07	0.00	0.11	0.01	0.10	0.01	0.004	0.018	0.782
18:2*n*-6	18.5^{a}	1.66	24.4^{b}	1.11	20.4^{a}	0.90	19.5^{a}	0.69	0.204	0.042	0.007
18:3*n*-3	1.29	0.10	0.92	0.03	1.23	0.06	0.78	0.04	0.126	<0.001	0.556
Σ CLA†	0.14^{a}	0.01	0.15^{a}	0.02	0.24^{b}	0.02	0.16^{a}	0.02	0.008	0.086	0.036
20:2*n*-6	0.14	0.01	0.18	0.01	0.12	0.01	0.17	0.01	0.167	<0.001	0.797
20:3*n*-9	0.29^{b}	0.02	0.19^{a}	0.01	0.26^{b}	0.02	0.25^{b}	0.02	0.392	0.003	0.005
20:3*n*-6	1.08^{a}	0.04	1.33^{b}	0.07	1.15^{ab}	0.06	1.14^{a}	0.05	0.297	0.036	0.026
20:4*n*-6	6.40	0.18	6.64	0.25	7.18	0.26	7.10	0.33	0.024	0.767	0.558
20:5*n*-3	0.61	0.02	0.42	0.04	0.69	0.05	0.50	0.04	0.060	<0.001	0.964

22:4*n*-6	0.52	0.01	0.58	0.04	0.53	0.04	0.62	0.04	0.440	0.054	0.714
22:5*n*-3	1.23	0.03	1.00	0.07	1.32	0.05	1.05	0.05	0.241	<0.001	0.709
22:6*n*-3	0.15	0.02	0.09	0.01	0.12	0.03	n.d.	.	.	0.007	.
Desaturation indices[‡]											
Δ^9-index 14	29.1	2.40	35.0	3.76	32.8	4.06	30.9	3.66	0.951	0.568	0.282
Δ^9-index 16	5.72^{bc}	0.39	3.84^{a}	0.30	5.36^{b}	0.29	6.33^{c}	0.29	0.002	0.165	<0.001
Δ^9-index 18	73.0^{ab}	1.61	70.5^{a}	1.28	69.2^{a}	1.38	74.0^{b}	1.00	0.934	0.417	0.010
Δ^9-index 18:1*t*11	25.0	1.46	22.7	1.33	28.2	2.45	23.4	2.46	0.339	0.087	0.533

Data are mean ± standard error (SE) for nine (AL-HS) or ten bulls (remaining groups) per treatment
Δ^9-index 14 = 100 × [*c*9-14:1/(14:0 + *c*9-14:1)]; Δ^9-index 16 = 100 × [*c*9-16:1/(16:0 + *c*9-16:1)]; Δ^9-index 18 = 100 × [18:1*c*9/(18:0 + 18:1*c*9)]; Δ^9-index 18:1*t*11= 100 × [18:2*c*9,*t*11/(18:1*t*11 + 18:2*c*9,*t*11)]
n.d. = not detected
[1]Post-hoc test revealed no significant differences between experimental groups, despite the significant interaction
[†]This sum is described in Table 5.4
[‡]Desaturation indices were calculated expressing fatty acid contents as nmol normalized to tissue weight
Means in the same row with different superscripts are significantly different (P<0.05)

Table 5.2 − Fatty acid concentration (mg/g fresh muscle) and centesimal distribution (mol% of total fatty acids) of fatty acids in neutral lipids of the *longissimus lumborum* muscle from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets.

	Alentejana				Barrosã				*P* value		
	HS		LS		HS		LS		Breed	Diet	Breed×Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total fatty acids	6.88[a]	0.72	8.21[a]	0.69	11.4[b]	0.98	21.4[c]	1.74	<0.001	<0.001	<0.001
Fatty acids											
14:0	3.46	0.15	3.52	0.15	3.59	0.12	3.71	0.19	0.303	0.558	0.857
14:1*c*9	0.43	0.04	0.51	0.05	0.60	0.04	0.66	0.08	0.007	0.228	0.894
16:0	29.5[c]	0.57	27.3[a]	0.50	28.0[ba]	0.38	28.9[bc]	0.30	0.811	0.148	0.002
16:1*c*9	3.10	0.20	3.18	0.16	3.66	0.15	3.76	0.20	0.004	0.627	0.935
18:0	17.2	0.71	15.3	0.54	14.7	0.42	14.4	0.54	0.005	0.064	0.156
18:1*t*6-*t*8	0.19	0.02	0.20	0.02	0.16	0.01	0.15	0.01	0.008	0.908	0.331
18:1*t*9	0.21	0.01	0.22	0.01	0.23	0.01	0.22	0.01	0.355	0.829	0.212
18:1*t*10	0.22[a]	0.01	0.69[c]	0.11	0.24[a]	0.01	0.31[b]	0.03	0.009	0.001	0.005
18:1*t*11	1.10	0.07	1.11	0.07	1.56	0.08	1.54	0.09	<.0001	0.952	0.919
18:1*c*9	32.2	0.59	32.3	0.86	33.9	0.54	33.6	0.19	0.020	0.868	0.717
18:1*c*11	3.02[a]	0.11	3.71[b]	0.11	3.99[bc]	0.12	4.18[c]	0.13	<.0001	0.001	0.039
18:1*c*12	0.55	0.03	0.64	0.04	0.70	0.03	0.77	0.04	0.000	0.029	0.695
18:1*c*13	0.22[a]	0.02	0.35[b]	0.02	0.34[b]	0.01	0.36[b]	0.02	0.003	0.001	0.006
18:1*c*14+*t*16	0.21	0.02	0.17	0.01	0.24	0.01	0.22	0.01	0.002	0.034	0.358
18:1*c*15	0.11	0.01	0.15	0.02	0.12	0.01	0.13	0.01	0.899	0.109	0.216
18:2*n-6*	1.74[a]	0.13	3.93[b]	0.67	1.86[a]	0.06	1.86[a]	0.13	0.019	0.010	0.010
18:3*n*-3	0.28	0.01	0.24	0.02	0.31	0.01	0.22	0.01	0.928	0.000	0.096
Σ CLA[†]	0.29	0.02	0.28	0.02	0.48	0.02	0.50	0.02	<0.001	0.877	0.327
20:4*n*-6	0.21[b]	0.04	0.71[c]	0.21	0.12[b]	0.01	0.07[a]	0.01	0.007	0.055	0.026
Desaturation indices[‡]											
Δ^9-index 14	10.9	0.81	12.4	0.97	14.3	0.71	15.1	1.52	0.007	0.280	0.749

Δ^9-index 16	9.47	0.51	10.5	0.56	11.5	0.35	11.5	0.52	0.004	0.342	0.299
Δ^9-index 18	65.2	1.11	67.8	1.02	69.8	0.83	70.0	0.81	0.001	0.143	0.227
Δ^9-index 18:1*t*11	16.5[a]	0.69	14.7[a]	0.71	20.0[b]	0.67	21.3[b]	0.82	<0.001	0.787	0.404

Data are mean ± standard error (SE) for nine (AL-HS) or ten bulls (remaining groups) per treatment
Δ^9-index 14 = 100 × [14:1*c*9/(14:0 + 14:1*c*9)]; Δ^9-index 16 = 100 × [16:1*c*9/(16:0 + 16:1*c*9)]; Δ^9-index 18 = 100 × [18:1*c*9/(18:0 + 18:1*c*9)]; Δ^9-index = 100 × [18:2*c*9,*t*11/(18:1*t*11+ 18:2 *c*9,*t*11)]
†This sum is described in Table 5.4
‡Desaturation indices were calculated expressing fatty acid contents as nmol normalized to tissue weight
Means in the same row with different superscripts are significantly different (P<0.05)

Table 5.3 − Total lipids (mg/g fresh muscle), fatty acid concentration (mg/g fresh muscle) and centesimal distribution (mol% of total fatty acids) of fatty acids in total lipids of the *longissimus lumborum* muscle from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana				Barrosã				*P* value		
	HS		LS		HS		LS		Breed	Diet	Breed×Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Total lipids	12.1[a]	0.76	12.5[a]	0.84	17.6[b]	1.21	27.6[c]	1.96	<0.001	<0.001	<0.001
Total fatty acids	11.1[a]	0.70	11.5[a]	0.77	16.2[b]	1.11	25. 5[c]	1.74	<0.001	<0.001	<0.001
Fatty acids											
14:0	2.52	0.14	2.76	0.15	2.80	0.09	3.30	0.12	0.003	0.008	0.324
14:1*c*9	0.34	0.03	0.45	0.04	0.50	0.03	0.60	0.07	0.004	0.044	0.881
16:0	25.9[a]	0.56	25.4[a]	0.45	25.4[a]	0.30	27.6[b]	0.31	0.053	0.049	0.005
16:1*c*9	2.63	0.2	2.79	0.11	3.21	0.11	3.57	0.2	<0.001	0.058	0.367
18:0	15.0	0.68	13.9	0.47	14.0	0.41	13.8	0.46	0.254	0.214	0.372
18:1*t*6-*t*8	0.11	0.01	0.13	0.01	0.11	0.01	0.12	0.01	0.528	0.097	0.515
18:1*t*9	0.16	0.01	0.20	0.03	0.20	0.02	0.20	0.01	0.378	0.266	0.375
18:1*t*10	0.15[a]	0.02	0.49[c]	0.07	0.30[abc]	0.11	0.31[b]	0.03	0.765	0.019	0.025
18:1*t*11	0.86	0.06	0.87	0.06	1.22	0.14	1.33	0.07	<0.001	0.541	0.617
18:1*c*9	28.3	0.77	28.8	0.74	30.7	0.85	33.0	0.67	<0.001	0.080	0.216
18:1*c*11	2.90	0.30	3.34	0.08	3.37	0.25	3.04	0.08	0.701	0.801	0.079
18:1*c*12	0.62	0.04	0.53	0.02	0.69	0.18	0.51	0.02	0.775	0.173	0.662
18:1*c*13	0.18	0.02	0.25	0.02	0.23	0.01	0.27	0.01	0.034	0.006	0.389
18:1*t*16+*c*14	0.16	0.02	0.12	0.01	0.19	0.01	0.18	0.02	0.001	0.107	0.321
18:1*c*15	0.08	0.01	0.08	0.01	0.10	0.01	0.11	0.01	0.049	0.731	0.669
18:2*n*-6	7.46[bc]	0.87	8.36[c]	0.74	6.05[b]	0.33	4.21[a]	0.38	<0.001	0.457	0.038
18:3*n*-3	0.62	0.05	0.42	0.02	0.54	0.03	0.29	0.01	0.006	<0.001	0.415
Σ CLA[†]	0.28	0.01	0.28	0.02	0.55	0.03	0.51	0.02	<0.001	0.429	0.402
20:2*n*-6	0.06	0.01	0.06	0.01	0.05	0.01	0.04	0.01	0.061	0.828	0.375
20:3n-9	0.15	0.02	0.09	0.01	0.07	0.01	0.05	0.01	<0.001	0.006	0.103

20:3*n*-6	0.40	0.03	0.37	0.02	0.27	0.03	0.16	0.02	<0.001	0.023	0.114
20:4*n*-6	2.25	0.23	2.05	0.15	1.63	0.12	0.95	0.13	<0.001	0.012	0.156
20:5*n*-3	0.22	0.03	0.14	0.01	0.14	0.02	0.08	0.01	0.001	<0.001	0.487
22:4*n*-6	0.21	0.01	0.22	0.01	0.14	0.01	0.10	0.01	<0.001	0.273	0.053
22:5*n*-3	0.51	0.06	0.44	0.05	0.46	0.10	0.18	0.02	0.026	0.012	0.108
22:6*n*-3	0.06	0.01	0.04	0.00	0.07	0.01	0.08	0.02	0.370	0.644	0.426
Other[‡]	5.53	0.36	4.83	0.21	4.62	0.19	3.37	0.22	<0.001	<0.001	0.287
Partial sums											
Σ SFA	44.8[ab]	1.03	43.7[a]	0.86	43.8[a]	0.51	46.2[b]	0.42	0.333	0.372	0.029
Σ cisMUFA	36.1	0.98	37.3	0.81	39.8	0.63	42.0	0.88	<0.001	0.047	0.550
Σ TFA	1.44	0.09	1.83	0.14	2.02	0.10	2.14	0.10	<0.001	0.027	0.226
Σ PUFA	11.9[ab]	1.22	12.2[a]	0.96	9.41[b]	0.55	6.07[a]	0.56	<0.001	0.088	0.050

Data are mean ± standard error (SE) for nine (AL-HS) or ten bulls (remaining groups) per treatment
Σ SFA = 14:0, 16:0 and 18:0; Σ MUFA = 14:1*c*9, 16:1*c*9, 18:1*c*9, 18:1*c*11, 18:1*c*12, 18:1*c*13 and 18:1*c*15; Σ TFA = 18:1*t*6-*t*8, 18:1*t*9, 18:1*t*10, 18:1*t*11 and 18:1 *t*16+*c*14; Σ PUFA = 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:3n-9, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-3 and 22:6*n*-3
[†]This sum is described in Table 5.4
[‡]The sum of the remaining area (others) includes dimethylacetals, branched chain fatty acids and unidentified peaks
Means in the same row with different superscripts are significantly different (P<0.05)

Table 5.4 − Centesimal distribution (mmol% of total fatty acids) of conjugated linoleic acid isomers in polar, neutral and total lipids of the *longissimus lumborum* muscle from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana				Barrosã				*P* value		
	HS		LS		HS		LS		Breed	Diet	Breed × Diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Polar lipids											
*t*7,*t*9	11.1	1.25	11.9	1.22	12.4	1.24	10.6	1.28	0.974	0.660	0.310
*t*6,*t*8	33.2^{a}	4.02	47.9^{b}	4.53	39.7^{ab}	4.89	27.2^{a}	3.99	0.117	0.791	0.004
*c*9,*t*11	86.3	7.87	89.6	12.5	149	21.3	102	15.1	0.020	0.161	0.107
*t*8,*c*10	2.00	0.17	1.39	0.41	5.02	2.97	2.06	0.45	0.262	0.275	0.461
*t*7,*c*9	11.4	2.44	7.08	0.87	21.3	4.76	25.8	2.75	<0.001	0.976	0.163
Neutral lipids											
*t*12,*t*14	3.31	0.70	2.62	0.35	3.66	0.60	2.47	0.18	0.847	0.073	0.623
*t*11,*t*13	4.84	0.69	2.82	0.36	5.48	0.48	4.80	0.21	0.011	0.009	0.165
*t*10,*t*12	4.56	1.03	3.36	0.30	3.87	0.91	3.87	0.35	0.908	0.419	0.420
*t*9,*t*11	5.06	1.12	5.37	0.39	8.30	1.73	8.30	0.46	0.010	0.886	0.887
*t*8,*t*10	1.89	0.60	4.46	1.66	2.75	0.69	1.51	0.13	0.292	0.495	0.066
*t*7,*t*9	5.61	0.84	5.30	0.87	3.64	0.46	2.53	0.35	0.002	0.304	0.559
*t*6,*t*8	9.54	1.52	6.64	0.80	2.59	0.54	2.66	0.19	<0.001	0.139	0.121
c/t12,14†	4.70	0.37	3.81	0.39	2.89	0.16	3.18	0.25	<0.001	0.339	0.067
*t*11,*c*13	7.54	0.83	3.07	0.24	9.34	0.89	6.26	0.35	<0.001	<0.001	0.293
*c*11,*t*13	1.02	0.09	1.42	0.14	1.58	0.20	2.23	0.26	0.001	0.009	0.510
*t*10,*c*12	3.62^{a}	0.36	7.63^{c}	0.86	4.68^{b}	0.35	5.86^{bc}	0.55	0.539	<0.001	0.022
*c*9,*t*11	218	16.7	189	13.8	388	22.4	412	19.5	<0.001	0.905	0.156
*t*8,*c*10	6.31	0.55	10.1	3.82	10.5	1.13	9.19	0.75	0.440	0.557	0.241
*t*7,*c*9	22.8	1.87	38.1	3.79	35.5	1.63	42.9	2.60	0.003	<0.001	0.146
*c*9,*c*11	4.26	0.36	4.44	0.45	4.88	0.27	5.18	0.28	0.062	0.495	0.877

Total lipids

*t*12,*t*14	1.90	0.17	1.72	0.37	2.92	0.29	2.38	0.27	0.006	0.225	0.532
*t*11,*t*13	4.27	0.42	2.87	0.33	7.18	0.34	4.75	0.31	<0.001	<0.001	0.155
*t*10,*t*12	3.40	0.83	4.34	0.62	4.29	0.67	3.86	0.49	0.755	0.706	0.311
*t*9,*t*11	7.61[a]	0.67	9.69[bc]	0.72	13.6[c]	1.68	9.07[b]	0.54	0.016	0.234	0.004
*t*8,*t*10	4.28	0.51	3.65	0.45	4.68	0.36	3.65	0.37	0.639	0.061	0.640
*t*7,*t*9	3.99	0.42	6.23	0.85	7.63	2.57	4.71	0.50	0.462	0.812	0.088
*t*6,*t*8	1.59[b]	0.17	0.83[a]	0.15	4.03[c]	0.46	0.92[a]	0.10	<0.001	<0.001	<0.001
c/t12,14[†]	1.67	0.14	1.85	0.23	1.91	0.18	1.95	0.19	0.362	0.580	0.699
*t*11,*c*13	7.07	0.60	3.58	0.37	11.0	1.19	6.62	0.47	<0.001	<0.001	0.546
c*11,*t*13*	1.59	0.28	2.33	0.38	2.50	0.27	2.61	0.28	0.060	0.178	0.306
*t*10,*c*12	7.36	1.87	9.56	1.55	6.50	1.10	5.81	0.82	0.111	0.592	0.309
*c*9,*t*11[§]	210	11.4	200	14.2	447	24.9	446	18.3	<0.001	0.771	0.812
*t*7,*c*9	24.4[a]	2.17	32.8[b]	3.13	29.1[ab]	2.32	17.0[a]	5.18	0.118	0.603	0.007
*c*9,*c*11	3.42	0.21	3.58	0.22	5.35	0.40	5.54	0.32	<0.001	0.559	0.956

Data are mean ± standard error (SE) for nine (AL-HS) or ten bulls (remaining groups) per treatment
[†]These fatty acids co-eluted
[§]This CLA isomer co-eluted with minor amounts of the *t*8,*c*10 isomer
Means in the same row with different superscripts are significantly different ($P<0.05$)

5.3.4 Meat total fatty acids

The contents of the main fatty acids and their partial sums in the LL muscle are shown in Table 5.4. The 18:1*c*9 (28-34% of total FAME), 16:0 (23-25%), 18:0 (14-15%) and 18:2*n*-6 (4-8%) fatty acids were the most abundant in meat. The percentages of 14:0, 14:1*c*9, 16:1*c*9, 18:1*t*11, 18:1*c*9, 18:1*c*13, 18:1*t*16 plus -*c*14, 18:1*c*15 and total CLA were higher in Barrosã when compared to Alentejana bulls ($P<0.05$). The Alentejana bulls had higher percentages of 18:3*n*-3, 20:3n-9, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6 and 22:5*n*-3 than the Barrosã bulls ($P<0.01$). Consequently, the meat from Alentejana bulls had lower percentages of MUFA and TFA than Barrosã bulls ($P<0.001$). Bulls fed the low silage had higher 14:0, 14:1*c*9 and 18:1*c*13 percentage than those fed the high silage diet ($P<0.01$). The percentages of 18:3*n*-3, along with most of the long chain PUFA were consistently higher in the high silage fed bulls when compared to those fed the low silage diet ($P<0.05$).

The Barrosã bulls had the highest 16:0 percentages and total SFA when fed the low silage diet, whereas in Alentejana bulls there was no differential response to dietary silage level (breed×diet, $P<0.05$). The 18:2*n*-6 percentages were similar in both Alentejana groups, whereas in Barrosã bulls the highest values were found in those fed the high silage diet (breed×diet, $P<0.05$).

5.3.5 Meat conjugated linoleic acid isomeric profile

Table 5.5 presents the percentages of individual CLA isomers in polar, neutral and total lipids of LL muscle. The *c*9,*t*11 and *t*7,*c*9 CLA isomers were higher in Barrosã than in Alentejana bulls ($P<0.05$). In Alentejana bulls, the *t*6,*t*8 CLA isomer was the highest when the low silage diet was provided, whereas in Barrosã bulls the opposite effect was found (breed×diet, $P<0.001$).

The CLA isomeric profile in the NL fraction (Table 5.5) was extensively influenced by breed. The main isomers in the NL fraction were *c*9,*t*11, *t*7,*c*9 , *t*11,*c*13 and *t*6,*t*8. The PL from Barrosã bulls had higher *t*11,*t*13, *t*9,*t*11 , *t*11,*c*13, *c*11,*t*13, *c*9,*t*11 and *t*7,*c*9 percentages than that from Alentejana bulls ($P<0.05$). In contrast, Alentejana bulls produced meat with higher *t*7,*t*9, *t*6,*t*8 and *c*/*t*12,14 percentages when compared to Barrosã bulls ($P<0.01$). Feeding the high silage diet promoted higher *t*11,*t*13, *t*11,*c*13 and *c*11,*t*13 percentages than the low silage diet ($P<0.05$), whereas the highest *t*7,*c*9-CLA percentage was found in the animals fed the low silage diet ($P<0.001$). The proportion of the *t*10,*c*12 isomer was increased by the low silage diet in Alentejana bulls, whereas no response to diet composition was found for Barrosã bulls (breed×diet, $P<0.05$).

The percentages of *t*12,*t*14, *t*11,*t*13, *t*11,*c*13, *c*9,*t*11 and *c*9,*c*11 were consistently higher in the meat total lipids from Barrosã bulls when compared to Alentejana meat ($P<0.05$). The *t*11,*t*13 and *t*11,*c*13 isomers, were higher in high silage-fed animals than in those fed the low silage diet ($P<0.001$). The *t*9,*t*11 and *t*6,*t*8 CLA isomers were highest in the meat from Barrosã bulls, particularly in those fed the high silage diet (breed×diet, $P<0.01$). The highest *t*7,*c*9 percentage was found in the meat from Alentejana bulls fed the low silage diet, with a similar response to diet composition in Barrosã bulls (breed×diet, $P<0.01$).

Table 5.5 − Pearson correlation coefficients between fatty acid composition (mol%, desaturation indices and *SCD* expression levels in *longissimus lumborum* muscle of Alentejana and Barrosã bulls fed high or low silage diets

	SCD expression level
Total lipids	
Total fatty acids	0.33*
Σ *c*9-MUFA	0.32*
Σ TFA	0.41*
18:1*t*11	0.40*
18:1*c9*	0.33*
CLA (*c*9,*t*11)	0.35*
Δ^9-index 14	0.10
Δ^9-index 16	0.03
Δ^9-index 18	0.08
Δ^9-index 18:1*t*11	-0.02
Neutral lipids	
Total fatty acids	0.34*
Σ *c*9-MUFA	0.35*
Σ TFA	0.42**
18:1*t*11	0.41**
18:1*c9*	0.35*
CLA (*c*9,*t*11)	0.38*
Δ^9-index 14	0.17
Δ^9-index 16	0.06
Δ^9-index 18	0.15
Δ^9-index 18:1*t*11	0.06

MUFA, TFA, Δ^9-index 14, Δ^9-index 16, Δ^9-index 18, Δ^9-index 18:1*t*11: see Tables 5.2 and 5.3 for variables explanation
*$P<0.05$, **$P<0.01$

5.3.6 Muscle SCD gene expression and correlation analysis

A correlation analysis was performed using the amounts (mg/g muscle) of fatty acids, instead of their percentages (g/100 g total fatty acids), and the results are depicted in Table 5.6. Moderate positive correlations (0.3≤r≤0.7) were found between the *SCD* gene expression levels and MUFA (*r*=0.32), TFA (*r*=0.41), 18:1*t*11(*r*=0.40), 18:1*c*9 (*r*=0.33) and *c*9,*t*11 CLA (*r*=0.35) in total lipids, with similar results in the NL fraction but not in the PL fraction, where no significant correlations were found (data not shown). However, no association was found between the *SCD* gene expression level and any of the calculated indices of Δ^9 desaturase.

5.4 Discussion

Fat content of muscle has great impact on the proportion of its fatty acids due to the distinct fatty acid composition between NL and PL. In the present study, lipid classes were separated to assess breed and diet effects on PL and NL, following reports of a selective incorporation of fatty acids between both fractions (Jerónimo *et al.*, 2011; Alfaia *et al.*, 2009). As fattening proceeds, the increase in the amount of PL is negligible, whereas NL predominate (Wood *et al.*, 2008). In line with this, our results showed no significant variations between experimental groups in the PL fraction. In contrast, the variation in the NL fraction reflected the changes found in total lipids.

Breed is one of the main factors influencing beef quality traits, namely fat content and composition. Higher intramuscular fat contents have been reported for Barrosã meat (Alfaia *et al.*, 2007) when compared to Alentejana meat (Alfaia *et al.*, 2009). However, these two breeds are generally raised in quite distinct production systems. As expected, small-framed Barrosã bulls produced meat with higher total lipids and fatty acid contents than the large-framed Alentejana bulls. Most interestingly though, was the fact that meat total lipids and total fatty acids were similar in Alentejana bulls regardless of the diet, whereas in Barrosã bulls the low silage diet promoted higher intramuscular fat deposition than the low silage one. This interaction between breed and diet suggests that the widely accepted concept that high concentrate diets increase beef intramuscular fat (Wood *et al.*, 2008) is dependent on maturity/phase of bovine growth.

The increase in TAG fatty acids in Barrosã bulls was accompanied by a significant increase in MUFA proportions, along with the SFA and TFA percentages. Large, lean breeds have long been associated with leaner meat and thus lower levels of MUFA in comparison to small, early maturing breeds (Johnson, 1987; Pitchford *et al.*, 2002). It is likely that the different rates of *de novo* fatty acid biosynthesis are due to different activities of lipogenic enzymes. SCD is the key enzyme responsible for the endogenous biosynthesis of *c9* MUFA (mostly 18:1*c*9) and *c*9,*t*11 CLA (Griinari & Bauman, 1999). In addition, several studies have

proposed the *SCD* gene expression level as an indicator of terminal adipocyte differentiation (Martin *et al.*, 1999) and, consequently, of intramuscular fat accumulation (Taniguchi *et al.*, 2004; Smith *et al.*, 2009b; Hiller, Herdmann & Nuernberg, 2011).

In the present study, we determined the *SCD* gene expression and calculated the product/substrate fatty acid ratios (desaturation indices) used as an estimate of *SCD* activity. As expected, Barrosã bulls with higher total fatty acids and MUFA content than Alentejana bulls, also presented, in the NL fraction, higher *SCD* desaturation indices. However, no significant differences in *SCD* gene expression were detected. Nevertheless, positive significant correlations between *SCD* expression and fatty acid content (mg fatty acids in NL/g muscle) was observed, particularly for total fatty acids, MUFA, 18:1*c*9, and TFA, including 18:1*t*11, and *c*9,*t*11 CLA, indicating an association between *SCD* gene expression and increasing fatty acid deposition in muscle. Taniguchi and colleagues (2004) also found an association between *SCD* mRNA expression and MUFA content in bovine subcutaneous adipose tissue, which was confirmed in subsequent works (Dance *et al.*, 2009; Duckett *et al.*, 2009). However, the same association has not been reported for muscle (Dance *et al.*, 2009; Bartŏn *et al.*, 2011). Nonetheless, the *SCD* gene expression in the muscle tissues is influenced by diet composition, particularly *n*-3 PUFA contents as shown in the works by Archibeque, Lunt, Tume & Smith (2005), Deiuliis *et al.* (2010). In the present study, the higher *n*-3 PUFA content in the low silage diet, when compared to the high silage diet, might have not been enough to influence the *SCD* mRNA levels enough to produce statistical significant differences between dietary treatments. Still, the high silage fed bulls showed the lowest levels of *SCD* gene expression, concomitant with a tendency for lower 16:1*c*9 and 18:1*c*9 percentages in comparison to the low silage fed bulls. No correlations were observed between fatty acid contents in PL and *SCD* gene expression (data not shown), suggesting that *SCD* does not play a major role on the regulation of fatty acid composition of membrane phospholipids. In addition, desaturation indices followed the same trend of intramuscular fatty acids and MUFA differences between breeds, which are in line with other works reporting breed-related differences in the calculated desaturase indices for beef cattle (Shen *et al.*, 2007; Siebert *et al.*, 2003). However, we did not obtain any correlation between them and *SCD* gene expression. Other studies have also failed to obtain significant correlations between the SCD expression and activity and desaturation indices (Archibeque, Lunt, Tume & Smith, 2005).

Both 18:2*n*-6 and 18:3*n*-3 in tissues originate from diet after escaping the rumen metabolisation. The incorporation of PUFA in the PL is higher than in the NL fraction (Wood *et al.*, 2008; Noci, Monahan, French & Moloney, 2005). Consequently, it is expected that breeds with low intramuscular fat contents and, therefore, with high phospholipids proportions, have higher proportions of PUFA than breeds with high intramuscular fat (Wood *et al.*, 2008). Accordingly, under our experimental conditions, Alentejana meat had higher

percentages of PUFA in total lipids when compared to Barrosã beef, whereas the latter showed higher proportions of 18:1*c*9. The separation of the lipid fractions confirmed that the breed-dependent variation in PUFA proportions were in fact due to differences in meat total fatty acids contents. Furthermore, in the PL fraction, diet composition was the major factor determining the individual PUFA contents, in agreement with previous reports (Noci *et al.*, 2005). The effect of diet on PUFA composition of PL followed the expected trend, with the high silage diet (lipids higher in 18:3*n*-3) resulting in higher *n*-3 PUFA in PL. Nevertheless, a breed×diet interaction was observed, whereas Alentejana bulls fed low silage diet replaced part of the 18:1*c*9 by 18:2*n*-6 in their membrane PL. It is not clear what determines this increased 18:2*n*-6 metabolic availability and that of 18:1*t*10 and *t*10,*c*12 CLA in Alentejana bulls fed low silage diets.

The 18:0 and *trans* octadecenoates (mostly 18:1*t*11) and CLA (mostly *c*9,*t*11 CLA) are the main products of rumen biohydrogenation. Increasing dietary concentrate usually results in either decreased (Glasser, Schmidely, Sauvant & Doreau, 2008) or incomplete biohydrogenation, resulting in an accumulation of *trans* octadecenoates (Chilliard *et al.*, 1997). Moreover, diets low in forage and high in starch induce changes in the rumen microbial populations which tend to shift the pattern of major biohydrogenation intermediates towards 18:1*t*10 production (Griinari & Bauman, 1999). In these situations, as 18:1*t*11 is further metabolized endogenously by SCD into *c*9,*t*11 CLA, in contrast to 18:1*t*10, the meat CLA content is often depressed. Nevertheless, as the *trans* biohydrogenation intermediates are preferentially deposited in NL (Jerónimo *et al.*, 2010) the level of meat fatness affects its concentration in meat.

Interestingly, the dietary effects on biohydrogenation intermediates are much less pronounced than the breed effects. Increasing concentrate proportion in the diet did not affect the 18:1*t*11 and *c*9,*t*11 CLA of both NL and total lipids in meat, but resulted in lower proportions of some octadecadienoic acids in NL, like *t*11,*t*13 CLA, the co-eluted peak of *c*12,*t*14 and *t*12,*c*14 CLA and the sum non-conjugated *trans* isomers of linoleic acid (LA, *t*,*t*+*c*,*t*-18:2). All of these fatty acids are derived mostly from 18:3*n*-3 biohydrogenation (Bessa *et al.*, 2007) and its decrease can be explained by the lower concentration of 18:3*n*-3 in low silage than in the high silage diet. The expected effects of diet on 18:1*t*10 and *t*10,*c*12 CLA were limited to Alentejana bulls and reinforce the breed differences observed on rumen biohydrogenation intermediates deposited in muscle. In fact, our results showed that, in general, total biohydrogenation intermediates were higher in Barrosã when compared to Alentejana breed (data not shown), indicating that this was not only due to the higher muscle fatty acid content but also due to a breed effect on rumen biohydrogenation. This is particularly evident for 18:1*t*11 and *c*9,*t*11 CLA in all lipid fractions. Most interesting though is the interaction between breed and diet observed for the 18:1*t*10. Increasing concentrate proportion in the diet promoted an increase of the 18:1*t*10 percentage in total lipids for

Alentejana but not for Barrosã breed. The 18:1*t*10 and *t*10,*c*12 CLA proportions in NL fraction reinforce that the response in Barrosã bulls was lower when compared to Alentejana bulls. To the authors' knowledge, the occurrence of such strong breed effect on rumen biohydrogenation pattern, even if evaluated indirectly by meat composition, has not been previously described. It is not easy to anticipate the mechanism involved but the saliva production rate, rumination pattern, feeding behaviour, rumen size and kinetics, including the rumen retention time, might play a role in the process and should be investigated.

The findings reported herein identified important interactions between bovine genetic background and dietary forage:concentrate ratio on intramuscular fat and its composition. The differential response to diet composition between Alentejana and Barrosã bulls suggests distinct ruminal biohydrogenation patterns, as indicated by the intramuscular fatty acids contents, as well as the increased 18:2*n*-6 metabolic availability and of 18:1*t*10 and *t*10,*c*12 CLA in Alentejana bulls fed the low silage diet. Briefly, the increased dietary concentrate proportion enhanced the deposition of intramuscular fatty acids in Barrosã but not in Alentejana bulls. Therefore, this study suggests that the manipulation of meat fatty acid composition could be oriented towards particular fatty acids based on the bovine genetic background. Beef with increased CLA contents seems to be easily achieved in small-framed (precocious) animal breeds, such as Barrosã, given its preferential lipid deposition in the NL fraction. In contrast, leaner bovine breeds, like Alentejana, seem to be a more appropriated choice for the production of meat with increased levels of long chain PUFA.

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CHAPTER 6

Hepatic regulation of lipid metabolism

Is lipid metabolism in the liver of beef cattle influenced by breed and dietary silage level?

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ASH Costa participated in the tissue sampling and laboratory work and was responsible for the statistical analysis. ASH Costa also collaborated in the interpretation and discussion of the results and preparation of the manuscript.

Abstract

In ruminants, unsaturated dietary fatty acids are biohydrogenated in the rumen and are further metabolised in various tissues, including liver, which has an important role in lipid and lipoprotein metabolism. Therefore, manipulation of muscle fatty acid composition should take into account liver metabolism. In the present study, the influence of breed and diet on liver lipid composition and gene expression was investigated in order to clarify the role of this organ in the lipid metabolism of ruminants. Forty purebred young bulls from two phylogenetically distant autochthonous cattle breeds, Alentejana and Barrosã, were assigned to two different diets (low *vs*. high silage) and slaughtered at 18 months of age. Liver fatty acid composition, mRNA levels of enzymes and transcription factors involved in lipid metabolism, as well as the plasma lipid profile, were assessed. In spite of similar plasma NEFAlevels, liver TAG content was higher in Barrosã than in Alentejana bulls. Moreover, the fatty acid composition of liver was clearly distinct from the remaining tissues involved in fatty acid metabolism of ruminants, as shown by Principal Components Analysis. The hepatic tissue is particularly rich in α-linolenic acid and their products of desaturation and elongation. Results indicate that *DGAT1*, *ELOVL2*, *FADS1* and *FADS2* genes influence the fatty acid composition of the liver the most. Moreover, genes such as *DGAT1* and *ELOVL2* appear to

be more sensitive to genetic background than to dietary manipulation, whereas genes encoding for desaturases, such as *FADS1*, appear to be modulated by dietary silage level. Our results indicate that liver plays an important role in the biosynthesis of *n*-3 LC-PUFA. It is also suggested that dietary silage level influences the hepatic fatty acid metabolism in a breed-dependent manner, through changes in the expression of genes encoding for enzymes associated with the desaturation and elongation pathway. The importance of devising custom-made feeding strategies taking into account the genetic background is, therefore, stressed by the results from this experiment.

6.1 Background

Despite the predominant role of adipose tissue in ruminant's *de novo* fatty acid synthesis, the liver also plays an important role in ruminant lipid metabolism (Bauchart *et al.*, 1996). This organ carries out central metabolic functions in various aspects of lipid and lipoprotein metabolism, such as uptake, oxidation and metabolic conversion of NEFA, synthesis of cholesterol and phospholipids, and formation and secretion of specific classes of lipoproteins (Bauchart *et al.*, 1996). The ruminants' liver removes little or no TAG from blood lipoproteins (Bell, 1981). Uptake of NEFA is the predominant route by which fatty acids are supplied to the liver (Emery *et al.*, 1992) and, thus, plasma lipid fatty acid composition should influence the liver fatty acid metabolism and composition (Bell, 1981). Consequently, the regulation of these liver metabolic pathways may affect fatty acid deposition into lipids of ruminant products (Gruffat *et al.*, 2011).

Interest in *n*-3 long-chain PUFA (*n*-3 LC-PUFA) has increased since it was found that their consumption in most Western populations, particularly those of EPA and DHA, is sub-optimal for protection against the most prevalent chronic diseases (Prates & Bessa, 2009). In grazing ruminants, α-linolenic acid content of muscles increases with the concomitant increase in *n*-3 LC-PUFA contents (Noci *et al.*, 2005). In contrast, although the addition of linseed to ruminant diets (Raes *et al.*, 2004; Bauchart *et al.*, 2005) increases the α-linolenic acid content of muscles, the *n*-3 LC-PUFA levels stay unchanged or increase only slightly. In fact, Bessa *et al.* (2007) reported that lucerne supplementation with linseed oil promoted an increase in α-linolenic acid coupled with a decrease in *n*-3 LC-PUFA in lambs, when compared to the control diet (lucerne only). According to the authors, these results suggest the inhibition of α-linolenic acid metabolism by vegetable oils rich in *n*-3 PUFA. Therefore, the abundance of *n*-3 LC-PUFA in ruminants' tissues appears to depend not only on dietary *n*-3 PUFA but also on their endogenous synthesis via elongation and desaturation of dietary *n*-3 PUFA.

The biosynthesis of DHA from α-linolenic acid is performed through alternating steps of desaturation and elongation, followed by a final step of peroxisomal β-oxidation. This

metabolic pathway involves two desaturases (Δ^5 and Δ^6 desaturases), two elongases (elongases 2 and 5) and enzymes of the peroxisomal β-oxidation (Nakamura *et al.*, 2004). The activity of these enzymes is currently regarded as potential limiting steps in this biosynthesis, possibly in a tissue dependent manner. However, recent studies addressed the role of the liver in ruminants' lipid metabolism, either using *in vitro* experiments (Gruffat *et al.*, 2011) or *in vivo* assays (Cherfaoui *et al.*, 2012). These experiments raised some interesting clues on hepatic lipid metabolism, namely the extensive catabolism of α-linolenic acid (Gruffat *et al.*, 2011) and the low or negligible expression level of genes encoding for enzymes of fatty acid desaturation and elongation (Cherfaoui *et al.*, 2012). Therefore, the role of bovine liver, as a central metabolic organ, on lipid metabolism remains to be elucidated.

An experiment with 40 young bulls from two genetically diverse beef cattle breeds, Alentejana and Barrosã, fed either high (70% silage/30% concentrate) or low (30% silage/70% concentrate) silage diets was carried out by our group to study the breed and diet effects on lipid metabolism. In Chapters 3 and 4 it was shown that these breeds have a distinct response to the variation in dietary silage level, as assessed by the fatty acid composition and the mRNA levels of key lipogenic factors of the main fat depots and muscle. Bearing this in mind, as well as the studies by Gruffat *et al.* (2011) and Cherfaoui *et al.* (2012), we aimed to investigate whether the same breed-specific response to dietary silage level would be observed in the liver. For this purpose, the detailed fatty acid composition of liver from Alentejana and Barrosã bulls, in parallel to their mRNA levels of key genes associated with lipid metabolism, were determined.

6.2 Material and Methods

6.2.1 Animals and experimental diets

Details concerning the experimental design, animals and the composition of experimental diets are provided in Chapter 2, section 2.2.

6.2.2 Blood sampling

One week prior to slaughter, blood samples were collected from the tail vein and centrifuged (3000 rpm for 15 minutes at room temperature) to harvest heparinized plasma. Total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analysed using diagnostic test kits (Roche Diagnostics, Mannheim, Germany), in a Modular Hitachi Analytical System (Roche Diagnostics). VLDL-cholesterol and total lipids were calculated as

described by Friedewald *et al.* (1972) and Covaci *et al.* (Covaci *et al.*, 2006) formulas, respectively.
Plasma NEFA were quantified using the Free Fatty Acid Quantification Kit (Biovision Inc, Mountain View, CA, USA).

6.2.3 Sample collection

Immediately after slaughter, liver samples for gene expression analysis were collected, rinsed with sterile RNAse-free water solution, cut into small pieces (thickness of ~0.3 cm), stabilised in RNA Later solution (Qiagen, Hilden, Germany) and subsequently stored at –80 ºC. A second sample (approximately 50 g) was vacuum-packed and stored at –20 ºC, until lipid extraction and determination of fatty acid composition.

6.2.4 Total lipid content and fatty acid composition

Liver samples were lyophilised (-60 °C and 2.0 hPa), maintained exsiccated at room temperature and analysed within two weeks. Total lipids were extracted by the method of Folch *et al.* (1957), as described in chapter 2, section 2.2.
Fatty acids were then converted to methyl esters, extracted and analysed by GC as described in chapter 3, section 3.2

6.2.5 Total RNA isolation

Frozen tissue samples were homogenized with an Ultra-Turrax® homogenizer (IKA-Labortechnik, Staufen, Germany). Total RNA was extracted from liver samples and purified as described in chapter 4, section 4.2. Total RNA extracts were immediately analysed for quantity (OD260nm) and purity (OD260nm/OD280nm) (NanoDrop ND-2000c, Peqlab GmbH, Erlangen, Germany), and stored at –80 ºC and until further analysis.

6.2.6 Synthesis of complementary DNA

Single-stranded cDNA was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Each 20 µl RT reaction contained 1200 ng of RNA template, 50 nM random RT Primer, 1×RT buffer, 0.25 mM of each dNTPs, 3.33 U/µl multiscribe reverse transcriptase and 0.25 U/µL RNase inhibitor, at temperatures of 25 ºC for 10 min, 37 ºC for 120 min, and 85 ºC for 5 min cDNA aliquots were stored at –20 °C. Total liver RNA was reverse

transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied BioSystems) according to manufacturer's instructions.

6.2.7 Primer design and housekeeping gene stability evaluation

Forward and reverse primers were optimally designed to cover exon–exon junctions to account for alternative splicing when possible (Table 6.1). Primer sequences for *PPARA*, *SCD* and *SREBF1* are available in Table 4.1.
Primer 3 (http://frodo.wi.mit.edu/) and Primer Express software v3.1 (Applied Biosystems, Foster City, CA, USA) were used to design primers for candidate housekeeping and target genes, amplicon length was fixed to 70–150 bp. Sequences, amplicon length and reference sequences are summarised in Table 6.1.
Evaluation of gene-specific primers and selection of the housekeeping gene were performed as previously described (chapter 4, section 4.2.5). The target gene expression levels were calculated using the geometric mean of ribosomal protein S9 (*RSP9)* and succinate dehydrogenase complex subunit A (*SDHA)* as a normaliser.

6.2.8 Real time quantitative polymerase chain reaction

The RT-qPCR was performed with the StepOne Plus™ Real-Time PCR System, using the Power SYBR® Green master mix (both Applied Biosystems, Foster City, CA, USA). Reaction mixes of 6.25 μL Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 1 μL of forward and reverse primers (160 nM) and 1 μL of diluted cDNA (1:15) template were pipetted into MicroAmp™ optical 96-Well reaction plates and sealed with optical caps (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed on a StepOnePlus thermocycler (Applied Biosystems) at standard cycling conditions (see chapter 4, section 4.2.6).

6.2.9 Data processing

The PCR efficiency was calculated for each primer. The efficiency curves were used to assess accuracy, linearity and efficiency of the PCR reaction. All primer sets exhibited an efficiency ranged between 85 and 110% and the correlation coefficients were higher than 0.990. The relative expression levels were calculated as described in chapter 4, section 4.2.7.

Table 6.1 − Specifications of oligonucleotides used for RT-qPCR

Gene symbol	Full gene name	Acc. Number [1]	Primer pairs (5'-3')	Amplicon length
CPT1A	carnitine palmitoyltransferase 1A	XM_002699420.2	F: TTCTTCTGGGGTCTACGATTCC	119
			R: GATGTGCTTGCTGTCCCTCAG	
DGAT1	diacylglycerol O-acyltransferase 1	NM_174693.2	F: TTGGCAGGTAAGGCGGC	99
			R: GGGGGCGAAGAGGAAGTAGT	
ELOVL2	fatty acid elongase 2	NM_001083517.1	F: GTCTTCTTACATGATGACGCTGGT	72
			R: ATTGGCTTTTTCCGGTATGTCTGA	
ELOVL5	fatty acid elongase 5	NM_001046597.1	F: CCCTCTCGGTTGGTTGTATTTC	127
			R: GTGGTCCTTTTGGTGCTCTCTC	
FADS1	fatty acid desaturase 1	XM_002699285.2	F: GTGGGTGGACTTGGCCTG	103
			R: TGGGGCTTGTCTTCATGGTC	
FADS2	fatty acid desaturase 2	NM_001083444.1	F: CGGCAAGAAGAAGCTGAAATACCTG	92
			R: CTGCTCATCCCTTTGTATTTCCA	
FASN	fatty acid synthase	NM_001012669.1	F: ATGGCGTTCCACTCCTACTTCA	137
			R: CTCTCCTGCCACTGGGTCTC	
INSR	insulin receptor	XM_002688832.2	F:ACGCTGGTGGTGATGGAGTT	133
			R: TCTCTGCCGCCATCTGAATC	
RPS9[3]	ribosomal protein S9	NM_001101152.1	F: GAAGGTAATGCCCTGTTGCG	141
			R: CAGGCCCAGCTTGAAGACC	
SDHA[3]	succinate dehydrogenase complex subunit A	NM_174178.2	F: TGCAGGAAGGCTGTGAGAAGAT	100
			R: GTCTCCACCAGGTCAGTGTTCC	

[1] Entrez Gene, National Center for Biotechnology Information (NCBI)
[2] F: forward primer and R: reverse primer.
[3] housekeeping gene

6.2.10 Statistical analysis

Statistical analyses were carried out with the Statistical Analysis Systems software package, version 9.2, (SAS Institute, Cary, NC, USA). All statistical analyses were performed based on a 2 × 2 factorial arrangement of breed (Alentejana and Barrosã purebreds), diet (high and low silage diets) and their respective interaction. The variances were tested for heteroscedasticity and, for most variables, variance was found to be heterogeneous. Therefore, subsequent data analysis was performed in order to account for heterogeneous variance. The general Satterthwaite approximation was computed in a mixed-effects regression model (PROC MIXED), with breed, diet and their interaction as fixed effects.
The final dataset was analysed using the MIXED procedure of SAS with a model that included breed, diet and their respective interaction as independent variables. Results were expressed as mean ± standard error. Differences between groups were examined for statistical significance using the PDIFF option (Fisher's test). Differences were significant at $P<0.05$ and tendencies discussed at $P<0.10$. Pearson correlation coefficients were calculated using the CORR procedure of SAS.

6.3 Results

6.3.1 Body composition and plasma metabolites

The body composition parameters and plasma metabolites are depicted in Table 6.2. Initial and slaughter weights were higher for Alentejana when compared to Barrosã bulls ($P<0.001$). Liver total lipids content was higher in Barrosã than in Alentejana bulls ($P<0.01$). Both aminotransferases, AST and ALT, were higher in Alentejana than in Barrosã bulls ($P<0.05$). The high silage fed bulls had higher ALT plasma levels than those fed the low silage diet ($P<0.05$). Liver weight, when expressed relatively to carcass weight, was similar across experimental groups ($P>0.05$). All plasma lipid parameters analysed were similar, regardless of breed or diet ($P>0.05$). The ALP plasma levels were not influenced by breed or dietary silage level ($P>0.05$).

Table 6.2 − Liver parameters and plasma metabolites of Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana		Barrosã		*P* value		
	HS	LS	HS	LS	breed	diet	breed×diet
Liver parameters							
Normalized liver weight (% carcass weight)	2.08±0.061	1.95±0.128	2.08±0.052	2.01±0.063	0.701	0.226	0.662
Hepatic total lipids (mg/100 g liver)	2.99±0.114	2.96±0.079	3.29±0.095	3.43±0.146	0.002	0.620	0.448
Plasma lipid profile							
Total cholesterol (mg/l)	863.0±49.487	884.0±77.305	892.0±46.614	837.0±103.517	0.903	0.818	0.607
HDL-cholesterol (mg/l)	408.00±17.499	393.00±25.693	366.00±14.391	353.00±40.989	0.139	0.606	0.971
LDL-cholesterol (mg/l)	83.10±6.413	84.90±12.673	78.0±06.464	84.00±9.452	0.745	0.672	0.820
VLDL-cholesterol (mg/l)	35.00±4.592	35.20±2.070	34.00±2.422	36.80±2.984	0.925	0.640	0.685
TAG†(mg/l)	175.0±22.961	176.0±10.349	170.0±12.111	184.0±14.922	0.925	0.640	0.685
Non esterified fatty acids (mM)	0.06±0.017	0.06±0.014	0.06±0.017	0.03±0.006	0.219	0.250	0.447
Total lipids (mg/l)	3401.00±112.965	3444.00±159.751	3454.00±96.646	3358.00±209.352	0.914	0.862	0.650
Plasma hepatic markers							
AST (U/l)	83.00±3.599	99.70±15.493	71.30±3.239	67.20±3.735	0.021	0.464	0.236
ALT (U/l)	30.40±1.833	28.90±3.020	26.50±2.212	18.10±1.690	0.003	0.036	0.136
ALP (U/l)	198.60±37.207	173.40±25.805	218.10±25.115	200.10±25.715	0.430	0.461	0.902

6.3.2 Total lipids and fatty acid composition analysis

The detailed fatty acid composition of the subcutaneous and mesenteric adipose tissues, as well as that of LL muscle, were described in chapters 3 and 5. The liver total fatty acid content and composition is depicted in Table 6.3. Total fatty acids content was higher in the liver from Barrosã when compared to Alentejana bulls (P<0.01). There were breed determined differences in 5 of the 31 identified fatty acids, but diet had the most important role over the individual fatty acid percentages. Alentejana bulls showed consistently lower percentages of 18:1*t*11, *c*9,*t*11 CLA, 20:3*n*-9 and TFA, when compared to the Barrosã bulls, but higher 20:4*n*-6 and 22:6*n*-3 percentages (P<0.05). The high silage diet promoted the deposition of 14:1c9, 15:0, 18:3*n*-3, 20:5*n*-3, 22:5*n*-*3*, 22:6*n*-3, when compared to the low silage diet (P<0.05). In contrast, the percentages of 18:1*t*6-*t*8, 18:1*t*9, 18:1*t*10, 18.1*t*12, 18:1*c*11, 18:1*c*13 and 22:4*n*-6 were higher in the low silage fed bulls, when compared to those fed the high silage diet (P<0.05). Total *n*-3 PUFA and *n*-3 LC-PUFA were higher in the liver from the high silage fed bulls, when compared to those fed the low silage diet (P<0.001). The Barrosã bulls fed the low silage had the lowest total PUFA and *n*-6 PUFA percentages (breed×diet, P<0.05).

6.3.3 Gene expression analysis

Results from the gene expression analysis are shown in Figure 1. Breed influenced the mRNA levels of both *DGAT1* and *ELOVL2*, with higher values for the Barrosã when compared to Alentejana bulls (P<0.05 and P<0.01, respectively). In turn, the expression levels of *FADS1* were higher in low silage in comparison to high silage fed bulls (P<0.05). Similarly, the low silage diet tended to promote higher *FADS2* mRNA expression levels than the high silage diet, but only in the Alentejana bulls (breed×diet P=0.072). In addition, Alentejana bulls tended to have the highest *PPARA* gene expression levels when fed the low silage diet, whereas the inverse trend was found for Barrosã bulls (breed×diet, P=0.086). Neither breed nor diet influenced the expression of *CPT1A*, *ELOVL5*, *FASN*, *INSR*, *SCD* and *SREBF1* genes (P>0.05).

Table 6.3 − Total fatty acids and fatty acid composition of liver from Alentejana and Barrosã bulls fed high (HS) or low silage (LS) diets

	Alentejana				Barrosã				*P* value		
	HS	SE	LS	SE	HS	SE	LS	SE	Breed	Diet	Breed×Diet
Total fatty acids	1.98	0.02	1.98	0.02	2.05	0.02	2.04	0.03	0.007	0.816	0.858
Fatty acids											
14:0	0.49	0.04	0.46	0.05	0.51	0.05	0.58	0.07	0.204	0.701	0.425
14:1*c*9	0.29	0.02	0.20	0.05	0.28	0.02	0.19	0.02	0.795	0.008	0.852
15:0	0.23	0.01	0.18	0.02	0.22	0.02	0.21	0.01	0.573	0.039	0.229
16:0	9.59	0.45	9.43	0.55	9.42	0.32	11.35	0.90	0.154	0.150	0.091
16:1*c*7	0.23	0.01	0.25	0.02	0.24	0.01	0.30	0.05	0.266	0.192	0.494
16:1*c*9	0.53	0.07	0.54	0.07	0.55	0.05	0.78	0.12	0.126	0.161	0.163
17:0	1.08	0.02	1.05	0.05	1.07	0.05	0.96	0.04	0.244	0.084	0.311
17:1*c*9	0.31	0.03	0.29	0.02	0.29	0.01	0.31	0.03	0.910	0.907	0.555
18:0	33.66	0.49	33.57	0.96	33.24	0.51	33.16	1.09	0.614	0.919	0.997
18:1*t*6-*t*8	0.05	0.00	0.07	0.01	0.05	0.00	0.06	0.00	0.647	0.010	0.922
18:1*t*9	0.06	0.00	0.08	0.00	0.06	0.00	0.09	0.01	0.333	<0.001	0.382
18:1*t*10	0.07	0.00	0.16	0.02	0.07	0.00	0.11	0.01	0.068	<0.001	0.060
18:1*t*11	0.86	0.07	0.97	0.09	1.09	0.11	1.11	0.07	0.049	0.484	0.644
18:1*t*12	0.37	0.02	0.43	0.03	0.37	0.01	0.44	0.03	0.794	0.019	0.792
18:1*c*9	11.42	0.69	10.86	0.52	11.11	0.30	12.91	0.89	0.186	0.345	0.078
18:1*c*11	1.58	0.06	1.78	0.13	1.55	0.05	1.99	0.20	0.516	0.022	0.370
18:1*c*12	0.28	0.02	0.27	0.02	0.25	0.02	0.31	0.03	0.775	0.224	0.106
18:1*c*13	0.10	0.01	0.13	0.01	0.10	0.01	0.13	0.01	0.964	0.010	0.792
18:1*t*16+*c*14	0.10	0.01	0.10	0.01	0.09	0.00	0.11	0.01	0.873	0.567	0.197
18:2*n*-6	16.16	0.87	17.08	1.17	16.32	0.51	14.67	1.02	0.235	0.694	0.174
18:3*n*-3	1.24	0.08	0.80	0.06	1.21	0.05	0.73	0.06	0.465	<0.001	0.796
CLA (*c*9*t*11)	0.37	0.02	0.28	0.03	0.48	0.04	0.44	0.06	0.001	0.098	0.453
20:2*n*-6	0.30	0.03	0.30	0.04	0.30	0.02	0.25	0.03	0.362	0.446	0.312
20:3n-9	0.39	0.03	0.33	0.03	0.77	0.04	0.78	0.04	<0.001	0.462	0.336
20:3*n*-6	2.43	0.21	2.76	0.29	2.54	0.25	2.64	0.25	0.982	0.383	0.636
20:4*n*-6	9.24	0.18	9.39	0.23	9.00	0.19	7.96	0.56	0.022	0.196	0.088
20:5*n*-3	0.42	0.02	0.25	0.02	0.37	0.02	0.26	0.02	0.412	<0.001	0.279
22:4*n*-6	1.64	0.10	2.26	0.21	1.86	0.12	1.97	0.22	0.839	0.042	0.145
22:5*n*-3	2.93	0.14	2.60	0.14	3.03	0.07	2.11	0.21	0.204	<0.001	0.056
22:6*n*-3	1.28	0.10	0.79	0.06	1.06	0.05	0.70	0.08	0.044	<0.001	0.393
Other	2.23	0.09	2.25	0.12	2.36	0.09	2.31	0.11	0.359	0.852	0.743
Partial sums											
SFA	45.38	0.24	44.95	0.63	44.82	0.25	46.55	0.96	0.393	0.292	0.087
MUFA	14.83	0.83	14.43	0.75	14.50	0.38	17.03	1.28	0.205	0.231	0.106
TFA	1.89	0.11	2.07	0.15	2.21	0.16	2.36	0.12	0.033	0.233	0.896
PUFA	36.01^{b}	0.83	36.56^{b}	0.85	36.46^{b}	0.37	32.05^{a}	1.55	0.055	0.066	0.021
n-3 PUFA	5.87	0.25	4.43	0.20	5.67	0.12	3.79	0.31	0.082	<0.001	0.349
n-6 PUFA	29.75^{ab}	0.77	31.80^{b}	0.82	30.02^{ab}	0.31	27.48^{a}	1.30	0.030	0.778	0.015
n-3 LCPUFA	4.63	0.24	3.64	0.22	4.46	0.12	3.06	0.28	0.107	<0.001	0.368

n-6 LCPUFA	13.59	0.30	14.72	0.57	13.70	0.42	12.81	0.83	0.124	0.835	0.087

[1] Different superscripts differ at least $P<0.05$. SFA = sum of 12:0,14:0, 15:0, 16:0, 17:0, 18:0 and 20:0; MUFA = sum of 14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 18:1*c*12, 18:1*c*13, 18:1*c*15, 19:1 and 20:1*c*11; TFA = sum of 18:1*t*6-*t*8, 18:1*t*9, 18:1*t*10, 18:1*t*11, 18:1*t*12, 18:1*t*16+*c*14, 18:2*c*9*t*11 and 18:2*t*11*c*15; PUFA = sum of 18:2*n*-6, 18:3*n*-3, 20:3*n*-6 and 20:4*n*-6; BCFA = sum of *i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0 and *i*18:0
[2] Total fatty acids are expressed as g/100 g liver; fatty acid composition is expressed as mol% fatty acids±standard error (SE)
[3]HS: high silage; LS: low silage

6.3.4 Correlation analysis

The correlation analysis between genes and fatty acid percentages is depicted in Table 6.4. The *FADS1* and *FADS2* genes were shown as the most associated with fatty acid composition, along with *CPT1A* and *ELOVL5*. Positive correlations were found for the *CPT1A* expression level and the 14:0 (r=0.56), and 16:0 (r=0.60) percentages. The *CPT1A* gene was also positively correlated with the percentages of 16:1*c*9 (r=0.47), 17:1*c*9 (r=0.31), and 18:1*c*9 (r=0.42). Negative correlations were observed between the *CPT1A* gene and 20:2*n*-6 (r=-0.59) and 22:4*n*-6 (r=-0.32) fatty acids.

The expression levels of *DGAT1* showed a moderate positive correlation with the 20:3n-9 percentage (r=0.44). The percentages of 16:0 (r=0.44), 16:1*c*9 (r=0.37) and 20:3n-9 (r=0.40) showed positive correlations with the *ELOVL2* gene expression levels. A negative correlation was observed between *ELOVL2* mRNA level and the 17:0 percentage (r=-0.37). Expression of the *ELOVL5* gene was positively correlated with the percentages of 14:0 (r=0.40), 16:0 (r=0.60), 16:1*c*9 (r=0.51), 17:1*c*9 (r=0.39) and 18:1*c*9 (r=0.50). In addition, there were *ELOVL5* mRNA levels were negatively correlated with 18:0 (r=-0.40), 18:1*c*9 (r=0.33), 18:2*n*-6 (r=-0.32) and 20:2*n*-6 (r=-0.46).

The *FADS1* mRNA levels were positively associated with 14:0 (r=0.39), 16:0 (r=0.42), 16:1*c*7 (r=0.48), 16:1*c*9 (r=0.38), 17:1*c*9 (r=0.33), 18:1*c*9 (r=0.38), 18:1*c*11 (r=0.51), 18:1*c*13 (r=0.48), 20:0 (r=0.48), 20:3*n*-6 (r=0.50) and 22:4*n*-6 (r=0.72) fatty acids. In addition, *FADS1* showed negative correlations with 18:0 (r=-0.41), 18:2*n*-6 (r=-0.53), 18:3*n*-3 (r=-0.46) and 20:2*n*-6 (r=-0.34). The relative expression of *FADS2* was positively correlated with the percentages of 14:0 (r=0.34), 16:1*c*7 (r=0.44), 18:1*t*9 (r=0.40), 18:1*c*11 (r=0.40), 18:1*c*13 (r=0.39), 20:0 (r=0.68), 20:1*c*11 (r=0.38), 20:3*n*-6 (r=0.67) and 22:4*n*-6 (r=0.70). Negative correlations were found between the *FADS2* and 18:*n*-6 (r=-0.52) and 18:3*n*-3 (r=-0.50) fatty acids percentages. The 14:1*c*9 fatty acid was positively correlated with the *FASN* gene expression levels (r=0.42).

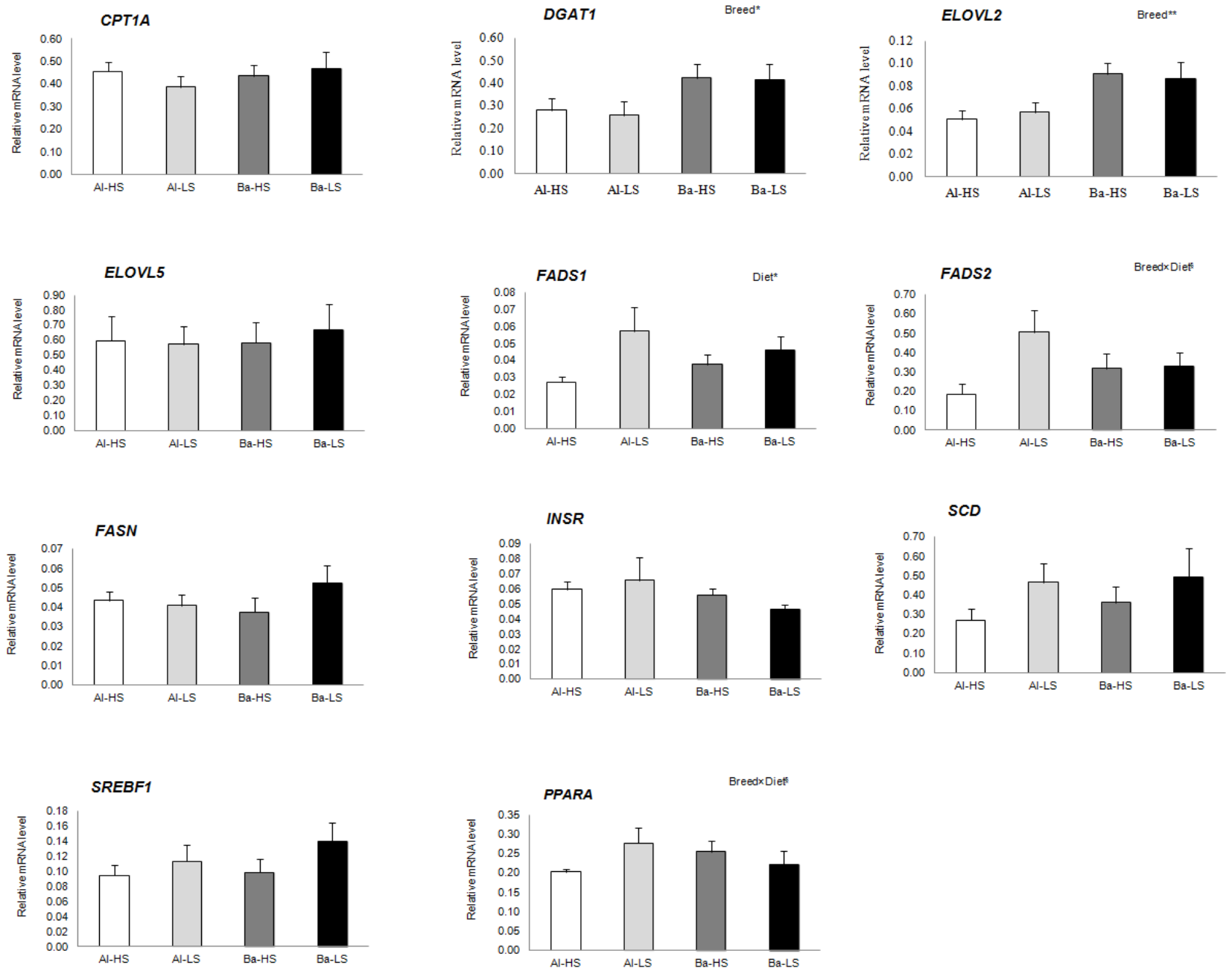

Figure 6.1 – Relative expression levels of the eleven selected genes in the liver from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low (LS) silage diets

Table 6.4 − Pearson correlation coefficients between the fatty acid composition and the relative gene expression levels in the liver from Alentejana and Barrosã bulls fed high or low silage diets.

	CPT1A	*DGAT1*	*ELOVL2*	*ELOVL5*	*FADS1*	*FADS2*	*FASN*	*INSR*	*PPARA*
14:0	0.56***			0.40*	0.39*	0.34*		0.32*	
14:1*c*9							0.42**		
16:0	0.60***		0.44**	0.60***	0.42**				
16:1*c*7					0.48**	0.44**			
16:1*c*9	0.47**		0.37*	0.51***	0.38*				
17:0			-0.37*						
17:1*c*9	0.31*			0.39*	0.33*				
18:0				-0.40**	-0.41**				
18:1*t*6*t*8						0.32*			
18:1*t*9					0.41*	0.40*			
18:1*t*10		-0.34*							
18:1*t*11									
18:1*t*12									
18:1*c*9	0.42**		0.33*	0.50**	0.38*				
18:1*c*11					0.51**	0.40*			
18:1*c*12									
18:1*c*13					0.48**	0.39*			
18:1*t*16*c*14									
18:2*n*-6				-0.32*	-0.53***	-0.52***		-0.33*	
20:0					0.48**	0.68***			
18:3*n*-3					-0.46**	-0.50**			
20:1*c*11						0.38*		0.32*	0.38*
20:2*n*-6	-0.59***			-0.46**	-0.34*			-0.38*	
20:3n-9		0.44**	0.47**						
20:3*n*-6					0.50**	0.67***		0.35*	
20:4*n*-6									0.34*
23:0									
20:5*n*-3									
22:4*n*-6	-0.32*				0.72***	0.70***			
22:5*n*-3								0.32*	

[1]Fatty acid contents expressed as mol/g liver
[2]There were no significant correlations between *SCD* or *SREBF1* and individual fatty acids

The *INSR* relative mRNA levels were positively correlated with the 14:0 (r=0.32), 20:1*c*11 (r=0.32), 20:3*n*-6 (r=0.35), 22:5*n*-3 (r=0.32) percentages, but negatively associated with the 18:2*n*-6 (r=-0.33) and 20:2*n*-6 percentages (r=-0.38). The PPARA mRNA levels showed positive correlations with 20:1*c*11 (r=0.38) and 20:4*n*-6 (r=0.34) fatty acids percentages.

6.4 Discussion

The present study was based on an experiment with two genetically different bovine breeds with distinct maturity rates, Alentejana and Barrosã, fed diets with different silage to concentrate ratio (30/70% *vs.* 70/30%). Alentejana and Barrosã breeds, despite being phylogenetically distant (Beja-Pereira, 2003) share more genetic similarities than the breeds used in previous studies addressing the differences between breeds in fatty acid metabolism, mainly based on the Japanese Black and Holstein breeds (Taniguchi *et al.*, 2004; Wang *et al.*, 2009; Albrecht *et al.*, 2011). We observed in these animals that different fat depots, subcutaneous and mesenteric adipose tissues, had distinct features regarding cellularity and fatty acid composition (Chapter 3). Results indicated that genetic background and, to a lesser extent diet composition, determine fat content and composition, pointing out to a differential fat partitioning between subcutaneous and intramuscular fat in Alentejana and Barrosã breeds. In addition, the comparison of fatty acid composition and gene expression levels between the muscle and subcutaneous adipose tissue indicated that these tissues play distinct roles in lipid metabolism, reinforcing the prevailing role of the subcutaneous adipose tissue over intramuscular fat in the *de novo* fatty acid synthesis (see Chapter 5).

The major metabolic fates of long chain fatty acid CoA in the liver are: i) esterification into TAG and, to a lesser extent, into phospholipids and cholesterol esters, ii) complete oxidation to CO_2 or incomplete oxidation which generates acetate and ketone bodies (Bauchart *et al.*, 1996). De La Torre *et al.* (2005) reported that, once incorporated *in vitro* into bovine hepatic cells, rumenic and oleic acids are highly catabolised through the β-oxidation pathway. One possible explanation might be the low efficiency of bovines in secreting fatty acids from the liver (Hocquette & Bauchart, 1999), thus directing fatty acids preferentially towards the oxidative pathway.

The liver is responsible for the uptake of NEFA and subsequent storage as TAG or release as VLDL. Hepatic TAG synthesis is the result of various pathways of lipid metabolism, including fatty acid uptake from plasma, oxidation of fatty acids, *de novo* synthesis of fatty acids and secretion of TAG via VLDL (Katoh, 2002; Schlegel *et al.*, 2012). Apart from hormones, the metabolic regulatory events in the liver are directed by metabolites levels, either in excess (*e.g.*, NEFA) or in shortage (*e.g.*, glucose) (Kreipe *et al.,* 2011). Once in the hepatocytes, catabolism of fatty acids is mostly directed towards the synthesis of ketone bodies for energy utilisation by tissues (Reid & Husbands, 1985). In spite of the higher insulin

levels observed in the bulls fed the low silage diet, when compared to those fed the high silage diet, it had no impact on *INSR* mRNA levels in the liver. Zhang *et al.* (Zhang *et al.*, 2011) reported low *INSR* gene expression levels in calf cultured hepatocytes in response to high insulin concentrations. However, it should be noted that in the present study even the highest insulin levels were within physiological levels.

Due to gluconeogenesis in liver, both propionate and lactate can indirectly modulate adipose tissue lipogenesis through increased glucose availability. The glucogenic effect of high-starch diets is often accompanied by an enhanced insulin response (Trenkle, 1978). The diacylglycerol acyltransferase (DGAT) catalyses the final step in TAG biosynthesis by converting diacylgycerols and fatty acyl-coenzyme A (CoA) into triacylglycero1s (Wang, Xu & Zhu, 2007). The *DGAT1* gene has also been related to increased hepatic TAG synthesis in dairy cows (Loor *et al.*, 2006). Moreover, mRNA levels of this gene appeared to be susceptible to diet-induced hyperinsulinaemia pre-partum (Loor *et al.*, 2006). In the present study, the relative expression levels of *DGAT1* was higher in the liver from Barrosã bulls, when compared to Alentejana bulls, although plasma insulin was affected by diet and not by breed.

It was suggested that bovine liver converts more efficiently LA into arachidonic acid than α-linolenic acid into *n-3* LC-PUFA (Kreipe *et al.*, 2011). In line with this, in the present study the ARA acid percentages were the highest amongst LC-PUFA. The PCA from the fatty acid pooled data showed a clear separation of the tissues analysed, with particular emphasis on the liver. LA and α-linolenic acid, along with ARA acid, were identified as the most contributing for the distancing of the liver cluster from those of the remaining tissues. This observation could be explained by the higher phospholipid/TAG ratio in the liver when compared to the other tissues. Given that LA and α-linolenic acid originate from diet, this suggests that the liver plays a role in their metabolism, possibly through their desaturation and elongation.

Attending to the putative role of liver in fatty acid elongation and desaturation, as described by others (Gruffat *et al.*, 2011; Cherfaoui *et al.*, 2012) and as suggested by the results herein presented for hepatic fatty acid composition, this work was focused on the analysis of gene expression levels of enzymes and transcription factors associated with the *n*-3 and *n*-6 LC-PUFA pathway synthesis. The PPARα, a transcription factor that acts as an important regulator of lipid metabolism and energy homeostasis, plays a key role in the control of the pathways involved in fatty acid uptake, fatty acid binding, fatty acid oxidation, ketogenesis, as well as carnitine synthesis (Desvergne & Wahli, 1999). The fact that the mRNA level of *CPT1A*, which is controlled by *PPARA*, was unchanged across experimental groups could indicate that there was no activation of *PPARA* in the liver. Moreover, activation of *PPARA* is known to be caused by increased plasma concentrations of NEFA. However, in the present

study, there were no significant changes in NEFA plasma levels, which is consistent with the similar *PPARA* levels across experimental groups.

Polyunsaturated fatty acids that escape β-oxidation could be converted into longer and/or more unsaturated fatty acids by the elongation-desaturation pathway, LA and α-linolenic acid being metabolised, theoretically, into 20:4*n*-6 and 22:6*n*-3, respectively (Sprecher, 2000; Burge & Calder, 2005). In a recent study by Gruffat *et al.* (2011), the conversion of α-linolenic acid into longer and/or more unsaturated fatty acids was not detected, yet 13.5% of LA was converted into ARA. It is widely accepted that members of the *n*-6 and *n*-3 families compete for the elongation-desaturation pathway (Sprecher, 2000). Furthermore, the conversion of α-linolenic acid and LA into their longer chain homologues is greatly determined by the composition of dietary fats (Christiansen *et al.*, 1991). Harnack, Andersen & Somoza (2009) suggested that a ratio of 1/1 would lead to the highest formation of *n-3* LC-PUFA, given that the conversion of *n-3* fatty acids into higher homologues may depend on the ratio of ingested *n*-6/*n*-3 fatty acids.

The carnitine palmitoyltransferase (CPT) system is an essential step in the β-oxidation of fatty acids, including LC-PUFA. Gruffat *et al.* (2011) showed that the rate of α-linolenic acid oxidation was higher than that of LA, possibly due to higher mitochondrial activity of CPT1A, as suggested by Ide *et al.* (1996). The positive correlations between *CPT1A* mRNA levels and the main SFA and MUFA, as well as the negative relationship with some PUFA (20:2*n*-6 and 22:4*n*-6) may be attributed to the role of the enzyme in fatty acid β-oxidation and ketogenesis. The higher SFA and MUFA percentages are a consequence of increased TAG and NEFA in the hepatic tissue, which would in turn promote fatty acid β-oxidation in order to prevent their excessive accumulation.

SREBF1 is a fundamental regulator of fatty acid biosynthesis, suggesting that it could be a key point of control of membrane lipid homeostasis capable of strongly influencing the lipid composition of membranes (Hagen, Rodriguez-Cuenca & Vidal-Puig, 2010). The *SREBF1* gene regulates a wide array of genes involved in lipid biosynthesis. In the present work, the *SREBF1* expression was shown to be correlated with the mRNA levels of *FASN*, *SCD*, *PPARA* and *INSR* (data not shown). The fatty acid synthase, encoded by *FASN*, plays a central role in *de novo* lipogenesis in mammals. However, the *FASN* mRNA levels were similar across experimental groups and showed no correlation with the main liver fatty acids, thus suggesting no modulation by diet or silage level under these experimental conditions.

The *SCD* gene encodes for an important enzyme in unsaturated fatty acid synthesis (2007). SCD activity in bovine liver and duodenal mucosal cells has been reported (Chang *et al.*, 1992; Archibeque *et al.*, 2005; Chung *et al.*, 2007). Furthermore, Chung *et al.* (2007) suggested that some portion of the MUFA in adipose tissues may arise from hepatic and mucosal desaturation of dietary SFA. However, in the present work, there was no apparent association between *SCD* gene expression levels and the desaturation indices. This finding

reinforces the concept of a low SCD activity in the liver, which is not to say that there is no desaturation at all. The matter of fact is that hepatic desaturation activity seems to be carried out mainly by the enzymes encoded by *FADS1* and *FADS2*.

The higher expression levels of *FADS1* and *FADS2* in the low silage than in the high silage fed bulls, particularly Alentejana bulls, is concomitant with the diet effect observed in the percentages of most LC-PUFA and, therefore, with the correlations found. That is, feeding the low silage diet, poorer in 18:2*n*-6 and α-linolenic acid than the high silage diet, promoted the expression of the genes encoding Δ^5 and Δ^6 desaturases in order to incorporate an adequate level of LC-PUFA in membrane phospholipids. The increase in the *FADS1* gene expression could have contributed to promote lipid biosynthesis and fatty acid deposition. The *FADS1* gene has been considered as one of the rate-limiting enzymes to the endogenous formation of LC-PUFA in humans (Lattka, Illig, Koletzko & Heinrich, 2010). In mammals, FADS1 converts dihomo-γ-linolenic acid (20:3*n*-6) to ARA and eicosatetraenoic acid (20:4*n-3*) to EPA with, respectively, LA and α-linolenic acid as the initial substrates (Schaeffer, 2006; Lattka *et al.*, 2010).

Liver seems to be highly active in α-linolenic acid catabolism (De La Torre *et al.*, 2005), thus limiting its subsequent availability for deposition in muscles. There are two basic metabolic fates for α-linolenic acid. First, it is subjected to β-oxidation and extensive carbon recycling. Second, it is converted into longer fatty acids via the elongation and desaturation pathway. Elongation of C18 in the LC-PUFA pathway occurs through elongase enzymes, which have been suggested to have a regulatory role on LC-PUFA synthesis and may be transcribed from one or more genes (*ELOVL2* and *ELOVL5*) (Jakobsson, Westerberg & Jakobsson, 2006). Hepatic LC-PUFA metabolism has been linked to energy balance and physiological state in dairy cattle. Long-chain fatty acyl elongases (ELOVL) are endoplasmatic reticulum membrane-bound proteins responsible for the first regulatory step in the fatty acid elongation pathway (condensation of activated fatty acids with malonyl-CoA), elongating fatty acid that are biosynthesized *de novo* or supplied by the diet (Morais *et al.*, 2009). In mammals, ELOVL2 has greatest activity in the elongation of C20 and C22 but low or, in the case of humans, no activity towards C18 PUFA (Leonard *et al.*, 2004). In contrast, mammalian ELOVL5 is very active towards C18 PUFA but does not appear to have the capacity to elongate beyond C22 (Leonard *et al.*, 2004; Inagaki *et al.*, 2002). Cherfaoui *et al.* (2012) proposed that the limiting step for elongation of α-linolenic acid in the muscle tissues is the absence of ELOVL5 protein. Moreover, these authors also suggested that the low levels of DHA in the muscle tissues could be a consequence of its preferential peroxidation or of its preferential uptake by other tissues.

Hepatic *ELOVL2* and *ELOVL5* are both regulated by *SREBP* transcription factors in mouse (Horton *et al.*, 2003). Nonetheless, in the present study there seemed to be no direct correlation between *SREBF1* mRNA levels and the expression of *ELOVL2* and *ELOVL5*

genes, in spite of *SREBF1* being correlated with total *n*-6 PUFA. The higher *ELOVL2* gene expression, and thus the higher fatty acid elongase with high activity in the final steps of LC-PUFA biosynthesis, in the Barrosã than in the Alentejana bulls could provide a route to promote EPA and DHA deposition. However, LC-PUFA contents in the liver from the present study were influenced, not by breed, but by diet.

In summary, the present study suggests that liver has, among the bovine lipogenic tissues, a specific role in lipid metabolism. In addition, the results indicate a breed modulation of hepatic desaturation/elongation of fatty acids, possibly through the differential expression of genes encoding for enzymes involved in the desaturation and elongation pathway. In some cases, the response to varying silage levels was modulated by the genetic background (*FADS2* and *PPARA*), whereas in others (*DGAT1* and *ELOVL2*) there was a clear breed effect regardless of diet composition. The small differences observed in gene expression levels could have additive effects, which may explain the differences found in the hepatic fatty acid profile, particularly in the LC-PUFA. In summary, the results herein discussed are in line with the previous reports from this experiment, thus stressing the importance of devising custom-made feeding strategies which take into account the genetic background.

CHAPTER 7

General discussion

Consumer preferences and the need to maximize the profitability of beef production systems led to carcasses with increased muscle proportion and decreased adipose tissue content. It is well established that large levels of subcutaneous and visceral fats, regarded as “waste fats”, are deposited in parallel to intramuscular, or “taste fat”, as maturing proceeds (Aldai *et al.*, 2007; Gotoh *et al.* 2009). Therefore, the manipulation of fattening in beef cattle is of major importance for the improvement of production efficiency, carcass composition and meat quality.

The adipose tissue is deposited in several anatomical locations: within the abdominal cavity (perirenal, mesenteric and omental), between the muscles (intermuscular), subcutaneously and within the muscles (intramuscular). Significant differences have been identified concerning the fat deposition process, particularly in what relates to intramuscular fat, and these have been related to animal of some of these breeds, namely Alentejana and Barrosã (Silva *et al.*, 1998; Alfaia *et al.*, 2006; Alfaia *et al.*, 2009). Nevertheless, the endocrine and genetic differences accounting for the contrasting lipid deposition between these breeds are not fully explored or understood. Therefore, this study aimed to provide fundamental knowledge to understand fat metabolism in beef cattle breeds with distinct genetic background, from a genetic and metabolic perspective.

7.1 Carcass composition and fat partitioning

Adipose tissue growth is accompanied by changes in the partition among the different depots due to their distinct lipid storage capacity (Robelin, 1986). Devising strategies to manipulate the levels of adipose tissue in carcass and non-carcass fat depots would allow for efficient carcass composition improvement. In order to do so, insights on the dynamics of fat deposition and partition are warranted. Recent studies addressing fat partitioning and deposition aiming carcass tissues in Alentejana and Barrosã bulls are rather scarce and lack the comparison between breeds and/or distinct diets (Simões *et al.*, 1995; Silva, 1996; Lemos, 1997). Therefore, the present study aimed to add further depth to the data already available about these two autochthonous bovine breeds.

In the present trial there was a significant effect of breed on carcass composition, which prevailed over the influence of diet composition. The results herein presented indicate that genetic background dictates the response to feeding strategy. In fact, deposition of adipose tissue in the visceral depots was enhanced in Barrosã bulls fed low silage diet when compared with those fed high silage diet. In contrast, fat deposition in Alentejana bulls appear to be less sensitive to the dietary silage level. A similar response pattern was found for intramuscular fat, but not for subcutaneous adipose tissue. Subcutaneous adipose tissue tended to be higher in Barrosã than in Alentejana bulls and decreased in the low silage fed

animals, when compared with those fed low silage diet. Thus, fat depot location is the third variable to take into account in order to manipulate adipose tissue accretion.

The effect of fat depot location could be explained, at least in part, by the circunstance that some depots, such as subcutaneous adipose tissue, tend to increase linearly, whereas others, as the intramuscular fat, might reach a plateau (May *et al.*, 1992; Duccket *et al.*, 1993).

7.2 Cellularity

Studies by Robelin (1981, 1986) and, more recently, by Mendizabal *et al.* (1999) and Eguinoa *et al.* (2003) identified breed and fat depot location as two major factors influencing adipocyte size. Given that lipogenic activity varies according to adipocyte size, it could be a factor contributing to depot specific differences in lipogenic activity. In addition, blood flow is not the same across the body. Indeed, Vernon (1980) and Gregory *et al.* (1986) reported that blood flow to abdominal sites is higher when compared with intramuscular or subcutaneous adipose tissues. Differences in blood flow translate into differences in nutrient supply and, therefore, this could influence the lipogenic activity of each of the tissues involved in the ruminants' lipid metabolism.

In the present study, adipocytes located in the SAT were found to be larger than those in the visceral fat (mesenteric adipose tissue). The differences between breeds regarding the expression of some adipogenic genes, as well as genes encoding fatty acid binding and transport proteins, found in SAT suggested that Barrosã bulls could have larger, more mature adipocytes than Alentejana bulls, but neither breed nor diet influenced the adipocytes' area. However, we must take into account that the differences observed in gene expression may preceed any noticeable change, or statistical difference, in adipocyte size. In fact, the average adipocyte area was higher in SAT of Barrosã bulls when compared with the Alentejana bulls. Moreover, the total lipid content was also highest in SAT of Barrosã bulls. Overall, the combined information from cellularity, gene expression and lipid content indicates that hypertrophy, and/or hyperplasia, was increased in the SAT from the Barrosã bulls in comparison with Alentejana bulls.

7.3 Fatty acid composition

The main fatty acids found in the tissues studied here (muscle, subcutaneous and mesenteric adipose tissues and liver) are depicted in Table 7.1. Fatty acid content was different among tissues, but also between breeds and, to a lesser extent, between diets. The

Table 7.1 – Total fatty acids (mg/g fresh tissue) and fatty acid composition (mg/100 g fresh tissue) of *Longissimus lumborum* muscle, subcutaneous adipose tissue (SAT), mesenteric adipose tissue (MAT) and liver from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets

	Liver				MAT				SAT				Muscle			
	AL-HS	AL-LS	BA-HS	BA-LS	AL-HS	AL-LS	BA-HS	BA-LS	AL-HS	AL-LS	BA-HS	BA-LS	AL-HS	AL-LS	BA-HS	BA-LS
Total fatty acids	1.98	1.98	2.05	2.04	603.48	557.71	441.29	531.83	496.28	473.16	455.44	436.47	9.33	10.13	12.67	21.87
Individual fatty acids																
14:0	0.75	0.72	0.82	0.92	1934	1724	1562	1718	1736	1635	1401	1395	19.59	22.93	29.30	60.39
*i*15:0	0.24	0.18	0.20	0.16	199	108	141	95.98	138	68.13	111	69.78	1.26	0.93	2.15	2.67
*a*15:0	0.21	0.19	0.19	0.17	279	181	185	165	123	88.70	108	78.72	1.51	1.36	1.89	2.45
14:1*c*9	0.44	0.31	0.45	0.30	82.41	76.00	71.93	90.10	352	366	440	493	2.62	3.72	5.03	10.87
15:0	0.38	0.29	0.37	0.35	413	327	313	269	209	230	213	170	2.64	2.97	3.95	5.92
*i*16:0	0.25	0.18	0.21	0.16	291	153	178	121	159	87.48	140	74.24	1.58	1.07	2.34	2.42
16:0	16.60	16.37	16.90	20.30	15539	14272	11553	13960	13551	12527	10957	10874	222	235	298	558
*i*17:0	0.29	0.27	0.30	0.29	213	180	163	169	140	115	143	111	1.99	1.86	2.95	4.32
16:1*c*7	0.39	0.42	0.43	0.52	239	195	168	179	127	122	143	124	2.43	2.22	3.15	4.31
16:1*c*9	0.92	0.92	0.97	1.39	597	556	448	613	2002	1771	2247	2314	22.86	25.67	36.41	73.71
*a*17:0	0.84	0.87	0.84	0.84	698	567	438	495	646	529	661	589	6.54	6.81	9.54	16.92
17:0	1.97	1.92	2.03	1.80	965	917	646	686	458	556	390	322	7.11	9.23	9.83	16.04
17:1*c*9	0.55	0.54	0.54	0.57	231	228	169	206	377	470	421	394	5.26	7.46	7.86	12.23
18:0	64.69	64.49	66.08	65.87	19502	17278	13018	14768	7268	6206	5300	4355	142	143	181	304
18:1*t*6+*t*8	0.10	0.13	0.10	0.13	112	136	90.17	123	57.74	86.01	63.07	68.81	1.00	1.47	1.40	2.78
18:1*t*9	0.11	0.15	0.12	0.17	153	158	114	152	114	117	109	118	1.52	2.29	2.50	4.58
18:1*t*10	0.13	0.31	0.13	0.21	165	539	122	236	97.28	471	117	177	1.50	5.19	3.46	7.11
18:1*t*11	1.66	1.85	2.15	2.18	1330	1229	1251	1483	683	661	876	775.23	8.15	9.00	15.80	30.05
18:1*c*9	21.75	20.76	21.95	25.48	12549	12045	9716	12958	16637	16484	16722	16250	268	295	398	745
18:1*c*11	3.01	3.41	3.05	3.92	1394	1391	1076	1480	2023	1730	1933	1981	27.78	34.39	43.42	68.71
18:1*c*12	0.53	0.51	0.48	0.61	365	348	263	361	377	334	356	366	5.86	5.44	8.42	11.64
18:1*c*13	0.19	0.25	0.21	0.26	67.41	72.43	52.96	77.72	137	178	178	222	1.71	2.61	3.08	6.20
18:1*t*16+*c*14	0.20	0.19	0.18	0.21	213	158	144	175	123	93.54	120	101	1.44	1.30	2.46	4.10
18:2*n*-6	30.71	32.33	31.98	28.81	1135	1506	859	1120	734	1082	783	796	64.36	80.85	74.23	86.73
20:0	0.25	0.27	0.28	0.31	108	96.74	84.57	90.27	46.42	38.61	36.87	27.10	1.15	1.17	1.54	2.02
18:3*n*-3	2.34	1.50	2.35	1.43	196	153	165	145	134	124	149	109	5.43	4.13	6.65	6.22

20:1*c*11	0.21	0.24	0.29	0.28	58.31	58.02	54.18	70.99	72.78	72.80	71.75	84.98	0.95	1.13	1.75	3.14
CLA (*c*9*t*11)	0.70	0.52	0.93	0.87	139	104	170	195	197	168	368	357	1.97	2.07	5.62	9.81
20:4*n*-6	19.00	19.33	19.18	16.99	27.24	32.12	22.83	25.19	15.65	16.07	22.26	20.03	21.62	21.65	21.44	20.19

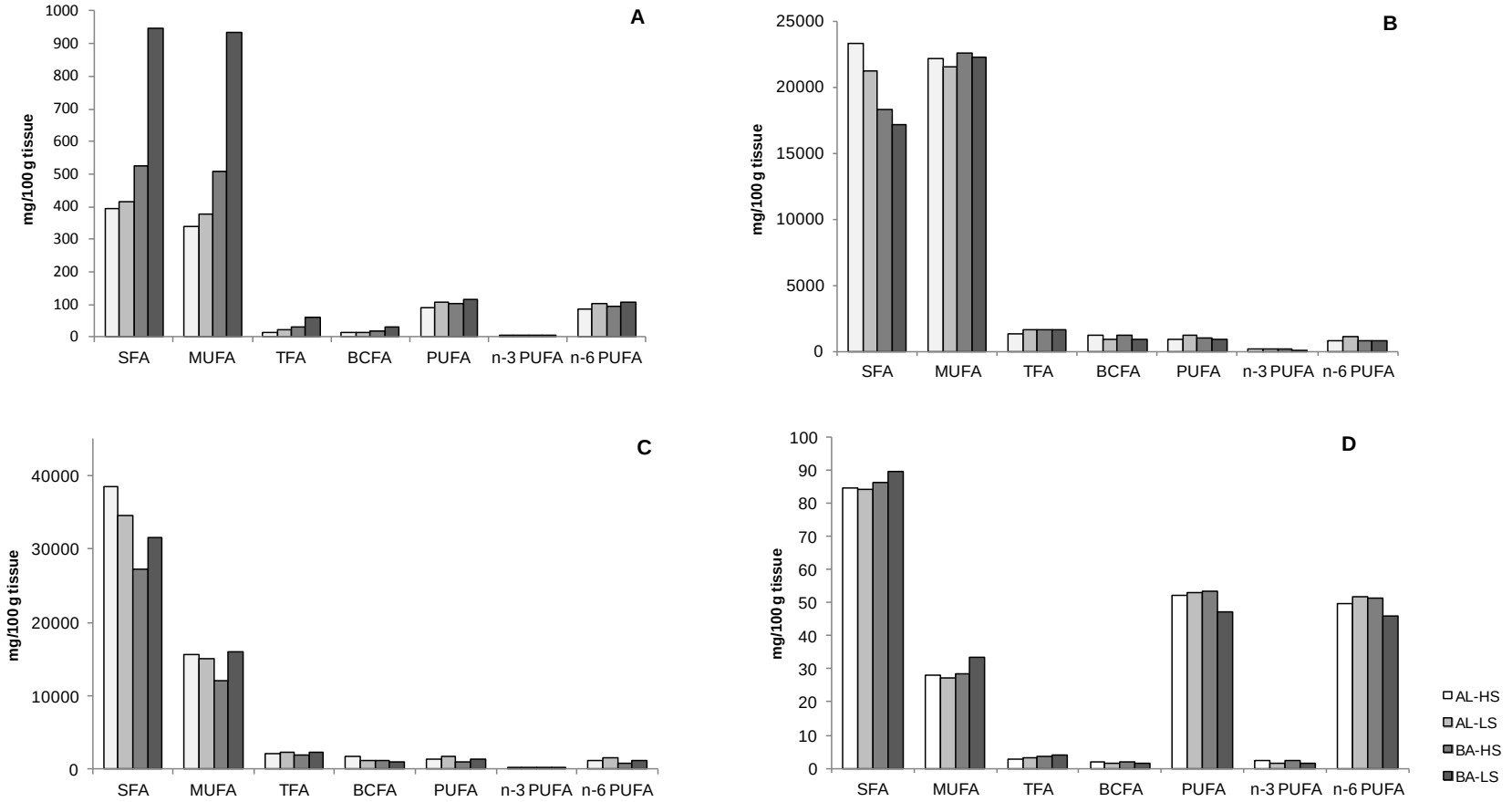

Figure 7.1 − Fatty acids contents (mg/100 g fresh tissue) in the *Longissimus lumborum* muscle (A), subcutaneous (B) and mesenteric adipose tissue (C), and liver (D) from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets

highest SFA contents were found in the mesenteric adipose tissue, followed by subcutaneous adipose tissue (Figure 7.1). The subcutaneous adipose tissue had also the highest MUFA contents, thus reinforcing the concept of being the main site for *de novo* fatty acid synthesis in bovines. Mesenteric adipose tissue had also the highest PUFA contents, although this particular group of fatty acids was the least abundant (2.3-3.1% of total fatty acids). LL muscle had equivalent SFA and MUFA percentages, in a pattern similar to the subcutaneous adipose tissue. In contrast, liver contents in PUFA, particularly in *n*-6 PUFA (21-36% of total fatty acids), surpass the contents in MUFA, being second only to SFA contents (43-44% of total fatty acids).

Observed differences in the fatty acid composition (percentage of total fatty acids) and content (weight per unit of tissue) are concomitant with the roles of each of the tissues analysed in the overall lipid metabolism, as reinforced by the PCA that follows. The IMF differs from the remaining fat depots, namely in the energy sources used. Indeed, the intramuscular adipocytes are reported to use glucose as the main energy source, whereas the adipocytes from the subcutaneous adipose tissue mostly use acetate (Rhoades *et al.*, 2007). Moreover, the intramuscular adipocytes are less sensitive to starvation than subcutaneous adipose tissues regarding TAG biosynthesis (Smith, Lin, Wilson, Lunt & Cross, 1998).

A PCA of the fatty acid composition parameters of all tissues studied (muscle, subcutaneous adipose tissue, mesenteric adipose tissue and liver) was performed in order to assess the distinctive metabolic features of each one and to describe the variability of the pooled data into two dimensions (Figure 7.2). The score plot of the first two components explains 74.6% of the total variability, with 48.13% for PC1 and 26.46% for PC2 (Table 7.2). The variables that contributed the most for PC1 were 14:0, 16:0, *a*17:0, 18:2*n*-6 and 20:4*n*-6. The MUFA 14:1*c*9 and 16:1*c*9, as well as the SFA 17:0 and 20:0, were the variables showing a greater contribution to PC2. Finally, 18:1*t*10 had a higher contribution to PC3.

The score plot from the PCA showed that fatty acids associated into four main clusters. In quadrant *b)* it is possible to observe a cluster formed by BCFA (*a*15:0, *i*15:0, *i*16:0, *i*17:0) and some 18:1 isomers (18:1*t*16 and 18:1*c*14 coeluted, and 18:1*t*6 to *t*8). A second cluster, with a significant contribution to PC1, was formed by 14:0, 16:0, *a*17:0, 18:1-*t*9 and 18:1*t*12. In quadrant *d)*, a cluster was formed by *c*9*t*11 CLA, 20:1*c*11, and some MUFA (14:1*c*9, 16:1*c*9, 17:1*c*9 and 18:1*c*11). Finally, 18:2*n*-6, 18:3*n*-3 and 20:4*n*-6 formed a cluster in quadrant *c)*, with little or no contribution to PC2 but with significant impact on PC1

.

Table 7.2 − Loadings for the first three principal components (PC).

Variable	PC1	PC2	PC3
14:0	0.935	0.049	-0.082
*i*15:0	0.659	0.470	0.447
*a*15:0	0.639	0.675	0.182
14:1*c*9	0.488	-0.731	0.190
15:0	0.791	0.507	0.034
*i*16:0	0.645	0.537	0.391
16:0	0.913	0.006	-0.178
*i*17:0	0.806	0.449	0.160
16:1*c*7	0.608	0.520	0.161
16:1*c*9	0.612	-0.748	0.108
*a*17:0	0.915	-0.121	0.217
17:0	0.287	0.800	-0.201
17:1*c*9	0.626	-0.680	-0.088
18:0	-0.520	0.772	0.071
18:1*t*6-*t*8	0.738	0.386	-0.366
18:1*t*9	0.895	0.112	-0.198
18:1*t*10	0.460	-0.026	-0.718
18:1*t*11	0.612	0.531	0.108
18:1*c*9	0.763	-0.560	-0.063
18:1*c*11	0.684	-0.581	-0.020
18:1*c*12	0.757	-0.243	-0.096
18:1*c*13	0.503	-0.757	0.032
18:1*t*16+*c*14	0.801	0.476	0.124
18:2*n*-6	-0.961	-0.015	0.040
20:0	-0.101	0.809	-0.063
18:3*n*-3	-0.858	0.051	0.225
20:1*c*11	0.233	-0.521	0.185
*c*9*t*11 CLA	0.272	-0.530	0.566
20:4*n*-6	-0.951	0.009	0.134
Proportion of variance (%)	48.13	26.46	0.06
Cumulative variance (%)	48.13	74.59	80.73

The score plot (Figure 7.2B) depicts the location of the four tissues in the multivariate space of the first two PCs. These scores were notably arranged in four clusters, corresponding to the muscle, liver and subcutaneous and mesenteric adipose tissues. The most notable result from this statistical approach is the separation between the cluster formed by the liver samples from the remaining clusters. PCA from the fatty acid pooled data showed a clear separation of the tissues analysed, with particular emphasis on the liver. The linoleic and linolenic fatty acids, along with arachidonic acid, were identified as being those that contributed for the distancing of the liver cluster from those of the remaining tissues. Given

that linoleic and linolenic acid originate from diet, this suggests that the liver plays a role in their metabolism, possibly through their desaturation and elongation. In addition, the liver is particularly rich in phospholipids and LC-PUFA, which are essential components of cellular membranes. It should also be noted that the cluster formed by the liver appears as the most homogenous when compared with muscle, SAT and MAT. Finally, it was possible to distinguish between Alentejana and Barrosã breeds in the cluster related to muscle.

PCA also allowed identifying the BCFA, as well as some biohydrogenation intermediates, as the fatty acids that contributed the most to distinguish the mesenteric adipose tissue from the remainder. The proximity from the digestive system, namely the small intestine, may facilitate a preferential deposition of the fatty acids arising from the rumen activity in the visceral fat depots. In fact, fatty acids originating from the diet have been reported to show a preferential accumulation in visceral adipose tissue depots rather than in intramuscular fat (Barber *et al.*, 2000; Eguinoa *et al.*, 2003).

In addition, MUFA appear as the class of fatty acids that contributed the most to separate SAT from the other tissues studied. Considering that subcutaneous adipose tissue is a major site for *de novo* fatty acid synthesis, mostly through desaturation, the results from the PCA were expected. The muscle fatty acid composition does not appear to be directly related to any particular class of fatty acids. A possible explanation to this result could be that the muscle is more active in the metabolisation of fatty acids than in their *de novo* synthesis.

The results from this statistical approach reinforce the differences previously described for the fatty acid contents and composition of these fat depots. The spacial distribution of the clusters formed by each of the tissues appears to arise from their roles in the metabolism of fatty acids. Therefore, mesenteric and subcutaneous adipose tissues are close to each other but in different quadrants and far from the cluster formed by the liver. In contrast, the liver is quite detached from the remaining tissues. Bearing in mind the fatty acid content and composition of these tissues and the PCA, one could propose that the mesenteric adipose tissue is mainly a storage site of TAG (mostly MUFA), whereas subcutaneous adipose tissue and, to a lesser extent, the muscle function as the sites for *de novo* synthesis of fatty acids. The hepatic tissue arises has the main site for LC-PUFA biosynthesis.

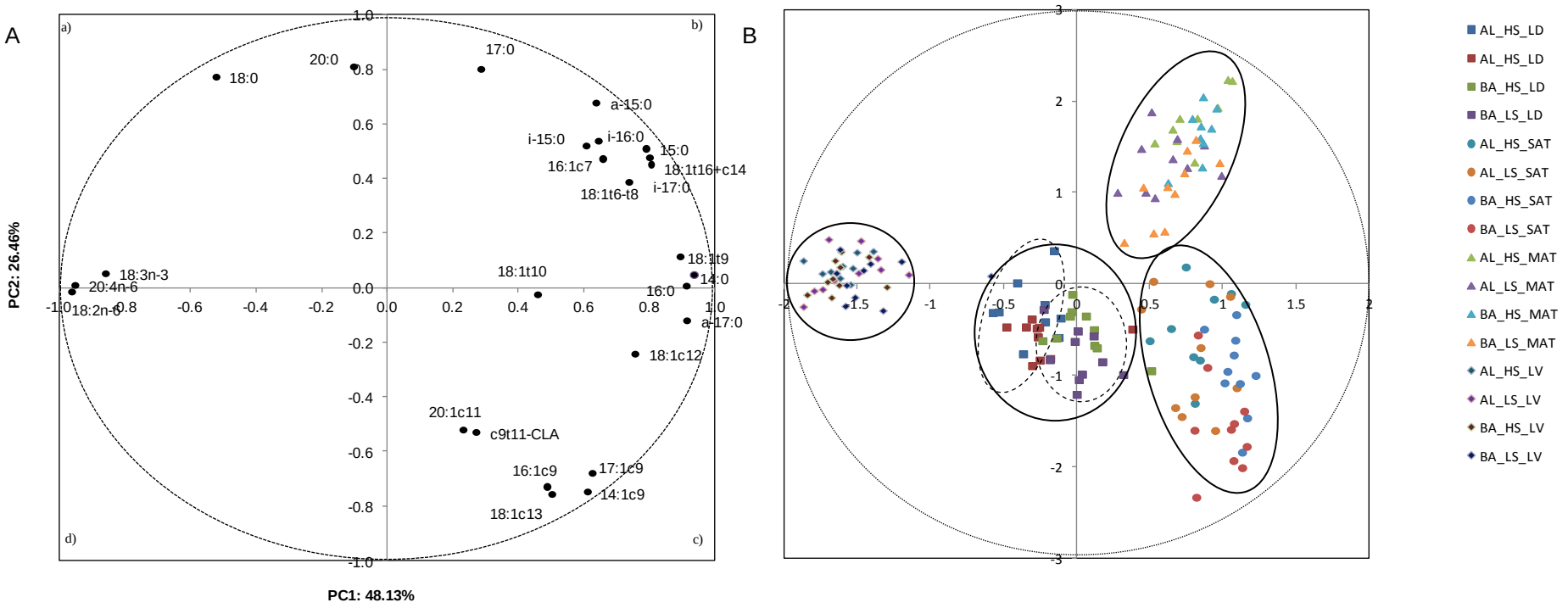

Figure 7.2 – Loading plot of the first and second principal components (PC) of the pooled data (A) and component's score vectors (B) for *longissimus lumborum* muscle (LL), subcutaneous adipose tissue (SAT), mesenteric adipose tissue (MAT) and liver (LV) from Alentejana (AL) and Barrosã (BA) bulls fed high (HS) or low silage (LS) diets.

7.4 Gene regulation of lipid metabolism

Levels of fat stored in subcutaneous, internal, and intramuscular adipose tissue depots are quantitative traits or complex phenotypes in nature, which are generally determined by the combined effects of many *loci* and are affected by genetic networks or molecular pathways (Dodson *et al.*, 2010). Specific genes can control and regulate adipocyte differentiation and metabolism.

Table 7.3 – Effect of breed and diet on gene expression levels of the tissues studied.

	Muscle	SAT	Liver
PPARA	ns	↑BA-LS	↑AL-LS
PPARG	↑BA breed	ns	–
SREBF1	ns	↑BA breed	Ns
		↑LS diet	Ns
INSR	–	–	Ns
FABP4	↑LS diet	↑BA-LS	–
LPL	↑HS diet	↑BA breed	–
DGAT1	–	–	↑BA breed
ACACA	↑LS diet	↑BA-LS	–
FASN	–	–	Ns
SCD	ns	↑BA breed	Ns
ELOVL2	–	–	↑BA breed
ELOVL5	–	–	NS
FADS1	–	–	↑LS diet
FADS2	–	–	↑AL-HS
CPT1	↑AL breed	ns	Ns
CRAT	ns	↑BA breed	–

–: not evaluated in this tissue; ns: no significant difference among experimental groups

Intramuscular adipocytes have been reported to accumulate TAG at a slower rate than subcutaneous adipocytes (Hood & Allen, 1978). Gondret *et al.* (2008) reported that expression levels of proteins involved in anabolic and energy-yielding catabolic pathways were lower in mature intramuscular adipocytes than in subcutaneous adipocytes. Dietary silage levels influenced the hepatic fatty acid metabolism in a breed-dependent manner through changes in the expression of genes encoding for enzymes associated with fatty acids desaturation and elongation pathways.

In the present work, SAT arose as the main site for *de novo* fatty acid synthesis and its gene expression levels were shown to be mostly influenced by the genetic background. In fact, PCA indicates that SAT mRNA expression profile allows for a better distinction between breeds than gene expression levels in muscle tissue. In addition, it is clearly shown in Table

7.3 that breed prevails over diet composition. Moreover, the levels of expression of genes analysed in the muscle and liver were shown to be more influenced by breed than by diet. The results from the present study have therefore suggested that there is a site-specific regulation of the lipogenic and adipogenic pathways, thus providing evidence for the possible manipulation of carcass fat partitioning

The importance of devising custom-made feeding strategies taking into account the genetic background is, therefore, stressed by the results presented here.

CHAPTER 8

General conclusion, implications and future perspectives

8.1 Conclusions

The results herein reported indicate that lipid deposition in intramuscular and visceral fat depots is higher in Barrosã than in Alentejana bulls. In fact, Barrosã bulls showed higher lipid deposition and are more sensitive to diet composition than Alentejana bulls. Neither breed nor diet had a significant influence on the cellularity of subcutaneous and mesenteric adipose tissue depots. Instead, there were significant differences between adipose tissue depots regarding the adipocytes' area number, which was lower in the subcutaneous fat depot than in the visceral adipose tissue depot.

The mesenteric adipose tissue was found to be the most saturated of all tissues studied. Mesenteric adipose tissue had also the highest PUFA contents, when compared with other tissues, although this class of fatty acids was the least abundant. The subcutaneous adipose tissue showed the highest MUFA content, in accordance with the theory of being the main site of *de novo* fatty acid synthesis in bovines. The LL muscle had equivalent SFA and MUFA percentages, in a pattern similar to the subcutaneous adipose tissue. In contrast, the liver contents in PUFA, particularly in *n*-6 PUFA, surpass the MUFA contents. The differences found in the fatty acid composition and contents are therefore in agreement with the putative roles of each of the tissues studied in the overall lipid metabolism.

For the particular case of the LL muscle, the differences between breeds in adiposity may be explained by higher adipogenesis in Barrosã bulls, induced by higher *PPAR*γ levels. Overall, muscle lipid metabolism, as evaluated by the expression of key adipogenic and lipogenic transcription factors and genes, appears to be more sensitive to dietary silage level than genetic background. However, the analysis of the LL muscle fatty acid composition showed that the influence of diet composition is modulated by the genetic background. This aspect might be explained by the effect of diet on the genes encoding fatty acid binding and transport, such as *FABP4* and *LPL*. In addition, the genes associated with fatty acid β-oxidation also tended to show higher expression levels in the Barrosã bulls, which suggests that lipid turnover could be increased in this breed when compared with Alentejana breed.

Finally, the present study suggests that liver has, among bovine lipogenic tissues, a specific role in lipid metabolism. Results indicate a breed modulation of hepatic desaturation/elongation of fatty acids, which may be explained by the differential expression of genes encoding for enzymes involved in the desaturation and elongation pathways. The effect of the silage proportion in diet was modulated by genetic background for the adipogenic and desaturation pathways associated genes (*PPARA* and *FADS2*). Regarding the *DGAT1* and *ELOVL2* genes, which are related to the synthesis of TAG and elongation of fatty acids, there was a clear influence of breed, regardless of dietary silage level. The differences in gene expression levels possibly have additive effects, thus explaining the differences found in hepatic fatty acid profile, particularly in LC-PUFA.

8.2 Implications and future perspectives

The results from the present study indicate that, when conceiving diet-based strategies to modulate the lipid deposition and composition, breed or genotype is a variable to take into consideration. We now know that whenever the goal is to increase the IMF content, it is easier to do it in Barrosã than in Alentejana bulls. In addition, increased meat CLA content can to be easily achieved in the small-framed, precocious, Barrosã bulls, given its preferential lipid deposition in the NL fraction. However, an increase in IMF is accompanied by enhanced fat deposition in other fat depots, such as visceral fat, thus jeopardizing the productive traits like feed conversion and average daily gain. On the other hand, Alentejana breed has the advantage of depositing more PUFA, which is interesting to improve the nutritional quality of meat.

The adipose tissues collected in this study consisted of a mixture of different cell types. Therefore future studies using bovine adipocyte cell lines may further verify the essential regulatory pathways of bovine adipogenesis and the depot-specific mechanisms of adipogenesis and lipogenesis regulation.

Altered microRNA expression in bovine subcutaneous and visceral adipose tissues from cattle under different diets has been reported (Romão *et al.*, 2012). Correlations between some microRNA and backfat thickness have also been established (Jin, Dodson, Moore, Basarab & Guan, 2010), thus suggesting a role for the microRNAs in ruminants' lipid metabolism. Still, the information regarding the role of the microRNAs remains scarce and warrants further studies. The modulation of lipid metabolism at the transcriptional level could also be assessed through the study of how proteins interact with DNA to regulate gene expression, namely using ChIP-Seq technology.

In addition, and regardless of the goal and strategy chosen, an integrated approach involving all major tissues/organs involved in the lipogenic process is warranted, given features and possibly metabolic functions of muscle, adipose tissues and liver. Currently, most studies on beef cattle lipid metabolism focus either on subcutaneous adipose tissue or intramuscular fat, thus neglecting visceral fat depots or other organs, such as the liver.

In order to address the complex ruminant lipid metabolism, there is a need to identify the transcript variations, not only in muscle tissue, but also in adipose and hepatic tissues that contribute to the reported breed and diet effects. The use of high throughput techniques would allow a global view of the complex interaction between ruminant tissues and fat depots. The interactions between networks of genes are of particular interest. A deeper understanding of the impact and regulation of those networks is the challenge which lies ahead for animal scientists.

Moreover, transcriptome expression patterns are not always correlated with the protein expression profiles, since biologically active proteins can be modified by the efficiency of translation, post-translational modification, and by the rate and extent of proteolysis (Dodson *et al.*, 2010). Consequently, it is of paramount importance to assess both transcriptome and proteome data in order to identify protein functions and to predict the molecular mechanisms regulating adipogenesis and lipid metabolism.

The environmental effects on meat quality are best defined as those not attributable to genetics, and include on-farm, *pre*-slaughter, and *post*-slaughter processing factors. It is recognised that environmental influences, *e.g. in utero* nutrition, can have effects beyond a single generation (Gluckman, Hanson & Beedle, 2007), which is the study of epigenetics. Epigenetics effects are potentially exciting new areas of research. Epigenetic mechanisms including DNA methylation, histone modifications and certain small RNA (sRNA) mediated pathways regulate gene expression, chromatin structure and genome stability.

Despite feeding strategy and diet composition being major factors influencing the fatty acid composition of ruminant-derived foods, genetic background cannot be neglected. In fact, further improvement can be expected through the use of genomic or marker-assisted selection so that the frequency of favourable genotypes may be increased, thus allowing the formulation of diets to take advantage of the genetic potential.

Bearing in mind that the present study showed that animals from distinct genetic background perform differently under the same diets, nutrigenomics and nutrigenetics, that is the interaction between nutrients and the metabolic pathways, is another approach that deserves further studies. In fact, the understanding of how diet composition impacts on genetic and epigenetic levels, altering gene expression or the structure of the genome would, contribute to conceive diets which would optimize animal performance.

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